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PACSIN2 and septin 7 in diabetic kidney disease: Roles in intracellular trafficking and actin cytoskeleton organisation in podocytes

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ACADEMIC DISSERTATION

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List of original publications

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I- Early-Onset Diabetic E1-DN Mice Develop Albuminuria and Glomerular Injury Typical of Diabetic Nephropathy
Mervi E. Hyvönen, Vincent Dumont, Jukka Tienari, Eero Lehtonen, Jarkko Ustinov, Marika Havana, Hannu Jalanko, Timo Otonkoski, Päivi J. Miettinen, and Sanna Lehtonen.
Biomed Research International, 2015, doi: 10.1155/2015/102969

II- PACSIN2 accelerates nephrin trafficking and is up-regulated in diabetic kidney disease
Vincent Dumont, Tuomas A. Tolvanen, Sara Kuusela, Hong Wang, Tuula A. Nyman, Sonja Lindfors, Jukka Tienari, Harry Nisen, Shiro Suetsugu, Markus Plomann, Hiroshi Kawachi, and Sanna Lehtonen.
The FASEB J, 2017, doi: 10.1096/fj.201601265R

III- PACSIN2 phosphorylation at serine 313 regulates actin cytoskeleton in podocytes and is elevated in diabetic kidney disease
Vincent Dumont, Rim Bouslama, Sonja Lindfors, Lassi Paavolainen, Jukka Tienari, Harry Nisen, Tuomas Mirtti, Carol Forsblom, Daniel Gordin, Per-Henrik Groop, Shiro Suetsugu, Sanna Lehtonen.
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IV- Septin 7 reduces nonmuscle myosin IIA activity in the SNAP23 complex and hinders GLUT4 storage vesicle docking and fusion
Anita A. Wasik, Vincent Dumont, Jukka Tienari, Tuula A. Nyman, Christopher L. Fogarty, Carol Forsblom, Markku Lehto, Eero Lehtonen, Per-Henrik Groop, and Sanna Lehtonen.
Experimental Cell Research, 2015, doi: 10.1016/j.yexcr.2016.12.010

Abbreviations

AER: albumin excretion rate
AGE: advanced glycosylation endproduct
BACER: Biochemical Analysis Core for Experimental Research
BSA: bovine serum albumin
CD2AP: CD2-associated protein
CDE: clathrin-dependent endocytosis
CIE: clathrin-independent endocytosis
CNS: congenital nephrotic syndrome
DKD: diabetic kidney disease
eGFR: estimated glomerular filtration rate
EGF-R: epidermal growth factor-receptor
EHD: Eps15 homology-domain-containing protein
eNOS: endothelial nitric oxide synthase
ESRD: end-stage renal disease
F-actin: filamentous actin
F-BAR: Fes-CIP4 homology Bin-Amphiphysin-Rvs161/167
FFA: free fatty acid
FSGS: focal segmental glomerulosclerosis
GBM: glomerular basement membrane
GFR: glomerular filtration rate
GLUT: glucose transporter
GSV: GLUT4 storage vesicle
IgG: immunoglobulin G
IL: interleukine
mTOR: mammalian Target Of Rapamycin
NF-κβ: nuclear factor-kappa beta
NMHC: nonmuscle myosin heavy chain
NMIIA: nonmuscle myosin II A
NPF: asparagine-proline-phenylalanine motif
N-WASP: neuronal Wiskott-Aldricht syndrome protein
O/N: over night
PACSIN: protein kinase C and casein kinase 2 substrate in neurons
PAS: periodic acid-Schiff
PB: phosphate buffer
PBS: phosphate buffered saline
PFA: paraformaldehyde
PI3K: phosphatidylinositol 3' kinase
PKC: protein kinase C
PLA: proximity ligation assay
pp-RLC: phosphorylated regulatory light chain
pS313-PACSIN2: PACSIN2 phosphorylated at S313
RLC: regulatory light chain
ROS: reactive oxygen species
RT: room temperature
SH3: Src-homology 3 domain

SHIP2: Src-homology 2-domain-containing inositol 5'-phosphatase 2
SIM: structured illumination microscopy
SNAP23: synaptosomal-associated protein 23
SNARE: soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors
SNP: nephrin overexpressing mouse podocytes
STZ: streptozotocin
T1D: type 1 diabetes
T2D: type 2 diabetes
TBS: tris buffer saline
TGF-β: transforming growth factor-alpha
TIRF: total internal reflection fluorescence microscopy
TNF-α: tumour necrosis factor-alpha
VAMP2: vesicle-associated membrane protein 2
VEGF: vascular endothelial growth factor
ZDF rat: Zucker Diabetic Fatty rat

Abstract

Background. Diabetes mellitus is a metabolic disease characterised by the inability of the body to maintain stable glycaemia. Diabetic kidney disease (DKD), the renal complication of diabetes, is the leading cause of end-stage renal disease in Europe and in the USA. Approximately 40% of individuals with diabetes develop this microvascular complication that may eventually require dialysis or kidney transplant. DKD is characterised by progressive loss of the permselectivity of the glomerular filtration barrier and declining glomerular filtration rate. The pathological mechanisms of DKD are not fully elucidated but podocyte injury is involved. In particular, alterations in the expression or localisation of nephrin, changes in the regulation of the actin cytoskeleton organisation and defects in the insulin signalling pathway are involved. This work characterises the renal phenotype of a novel transgenic mouse model of hyperglycaemia triggered by hypoinsulinaemia due to the defective β-cell mass growth in the pancreas. It also aims to evaluate the expression, the role and the phosphorylation status of protein kinase C and casein kinase 2 substrate in neurons (PACSIN2) in the trafficking of nephrin and the regulation of the actin cytoskeleton organisation in the context of DKD in podocytes. Further, the thesis refines how septin 7 hinders the trafficking of glucose transporter 4 (GLUT4) storage vesicles, as septin 7 was recently found to inhibit glucose uptake in podocytes.

Results. The characterisation of hyperglycaemic E1-DN mice revealed in homozygous mice an increased urine volume as well as albuminuria, the severity of which correlated with the hyperglycaemia. The kidneys developed changes typical of human DKD, such as tubular proliferation and atrophy, mesangial expansion and glomerular basement membrane thickening, lower expression of nephrin, foot process effacement and podocyte apoptosis.

Next, we found that PACSIN2 expression was elevated in the glomeruli of obese Zucker Diabetic Fatty (ZDF) rats, a model of advanced DKD. Interestingly, the obese ZDF rats had albuminuria and altered localisation of nephrin. We found that overexpression of PACSIN2 increased both endocytosis and recycling of nephrin. This may rely on the newly identified PACSIN2-nephrin-rabenosyn-5 protein complex. Interestingly, the interaction of PACSIN2 with nephrin was stimulated by treating podocytes with palmitate, the most abundant free fatty acid (FFA) in the circulation and elevated in diabetes.

Our data using isolated glomeruli from ZDF rats and human, as well as using cultured podocytes treated with sera from diabetic patients with normal kidney function or microalbuminuria suggested that PACSIN2 phosphorylation at serine 313 is increased in the context of DKD but not diabetes alone. We identified that palmitate induced the phosphorylation of PACSIN2 at S313 in a protein kinase C-dependent manner. Finally, we found that overexpression of PACSIN2 altered the actin cytoskeleton organisation and morphology of podocytes in culture, and that constitutively phosphorylated PACSIN2 at S313 showed milder effect.

In the work concentrating on the trafficking of GLUT4 in podocytes, we found that septin 7 forms a complex with nephrin and nonmuscle myosin IIA (NMIIA) at the plasma membrane in the docking and fusion site of GLUT4 storage vesicles (GSVs). We showed that removal of septin 7 and activation of NMIIA in the docking and fusion site are essential for insulin-stimulated glucose uptake. Also, the presence of septin 7 and the activity of NMIIA at the docking and fusion site appeared to be regulated by circulating factors in the serum of individuals with type 1 diabetes and macroalbuminuria.

Conclusion. In this thesis, we identified that PACSIN2, the expression of which is increased in podocytes in DKD, regulates the localisation of nephrin in cultured podocytes. *In vitro*, PACSIN2 also regulates the actin cytoskeleton organisation depending on its phosphorylation at S313, also elevated in DKD. We found that septin 7, together with NMIIA, regulates the docking and fusion of GSVs with the plasma membrane in podocytes. Thus, we propose that PACSIN2, septin 7 and NMIIA are central molecules in podocytes, and participate in the progression towards DKD by regulating the organisation of the actin cytoskeleton and the trafficking of nephrin and GLUT4.

1. Introduction

Diabetes mellitus is a chronic disease characterised by hyperglycaemia due to defective insulin production in the pancreas and the inefficacy of the insulin to trigger the cellular responses in the target tissues (also called insulin resistance). The loss of glycaemic control in people with diabetes damages blood vessels, as well as various organs, including eyes, nerves and kidneys (World Health Organization, www.who.int). The diagnosis of diabetes traditionally relies on the fasting glucose values and oral glucose tolerance test, but the reasons for the loss of glycaemic control are heterogeneous. Although treatments are available to slow the progression of the disease and its complications, there is currently no available cure for any of the subtypes of diabetes.

Diabetes is a pandemic. It used to be more prevalent in developed countries, but it now reaches virtually all human populations, and, currently, diabetes progresses faster in low- and middle-income countries compared to high-income countries. The number of individuals with diabetes has increased from 108 million in 1980 to 422 million in 2014. Therefore, the global prevalence of diabetes has increased in this period of time from 4.7% to 8.5% in adults over 18 years of age (World Health Organization). Moreover, recent projections suggest that the proportion of people with diabetes is likely to almost double in the USA by 2060 to reach 17.9% of the overall adult population (against 9.1% in 2014) (1). In line with this, the overall number of people with diabetes is expected to increase from 425 million in 2017 to 629 million in 2045, almost doubling worldwide in less than 30 years (*Diabetes Atlas*, 8th edition, International Diabetes Federation). Consequently, the prevalence of diabetic complications is likely to continue to rise and be an economical burden for societies in the coming decades.

Increased mortality and morbidity amongst patients with diabetes are mostly due to the complications. In particular, the long-term microvascular complication affecting the kidney, called diabetic kidney disease (DKD), occurs in approximately 40% of individuals with diabetes. The risk of developing the disease varies with the age at the onset of diabetes, the subtype of diabetes, as well as clinical characteristics and the lifestyle of the individuals (2-4).

In DKD, various renal cell types are affected, both in the tubules and in the glomeruli. This thesis project focuses on studying the changes occurring in the glomeruli, and specifically in glomerular visceral epithelial cells, also known as podocytes. Podocytes are highly differentiated and specialised cells. Their function relies on their architecture, which depends on well-organised actin cytoskeleton and controlled intracellular trafficking (5, 6). Moreover, the insulin signalling pathway has been shown to be essential for podocytes and kidney function (7). Here, we characterise the renal phenotype of a novel transgenic mouse model of hyperglycaemia. We also investigate how protein kinase C and casein kinase 2 substrate in neurons (PACSIN2), a known regulator of the actin cytoskeleton and endocytosis, and septin 7, recently identified as a regulator of glucose transporter trafficking in podocytes, participate in the maintenance of normal podocyte physiology and development of DKD.

2. Review of the literature

2.1. Kidney

2.1.1. Structure and function of the kidney

The main function of the kidneys is to keep a stable internal environment in the body by maintaining the plasma composition. Specifically, the kidney filters the plasma and forms urine, allowing clearance of some of the metabolic waste materials as well as maintenance of osmotic and acid-base equilibrium. In particular, the kidney regulates the excretion of water, potassium, sodium, phosphate, chloride, calcium and magnesium, while preventing the loss of proteins and glucose in healthy conditions (8).

For this, the kidney has developed a functional unit called nephron composed of a glomerulus, the site of ultrafiltration, and its adjacent tubule, where reabsorption and secretion of various substances lead to the formation of final urine (8). The number of nephrons is approximately 1 million per kidney in adults, but it can vary from 0.2–2 million with age and between individuals (9). Every day, 180 litres of plasma is filtered and most of the primary urine is reabsorbed to produce around 2 litres of final urine (8).

Anatomically, the kidneys are located in the abdominal cavity, behind the peritoneum and between the twelfth thoracic vertebra and the third abdominal vertebra. The blood supply enters the kidney *via* the renal arteries that originate from the abdominal aorta, and exits *via* the renal veins that lead to the inferior vena cava. As the kidney is a capsulated organ, the renal arteries and veins penetrate the kidney in the medial area called hilus. The outer part of the kidney, where the glomeruli are located, is called the cortex, while the loops of Henle and collecting ducts are in the inner part of the kidney, called medulla. The kidney is subdivided into pyramids. The newly formed urine in the nephrons flows from the apical part of the pyramids into the calyces, and exits the kidney *via* the renal pelvis to the ureter and bladder (8) (figure 1).

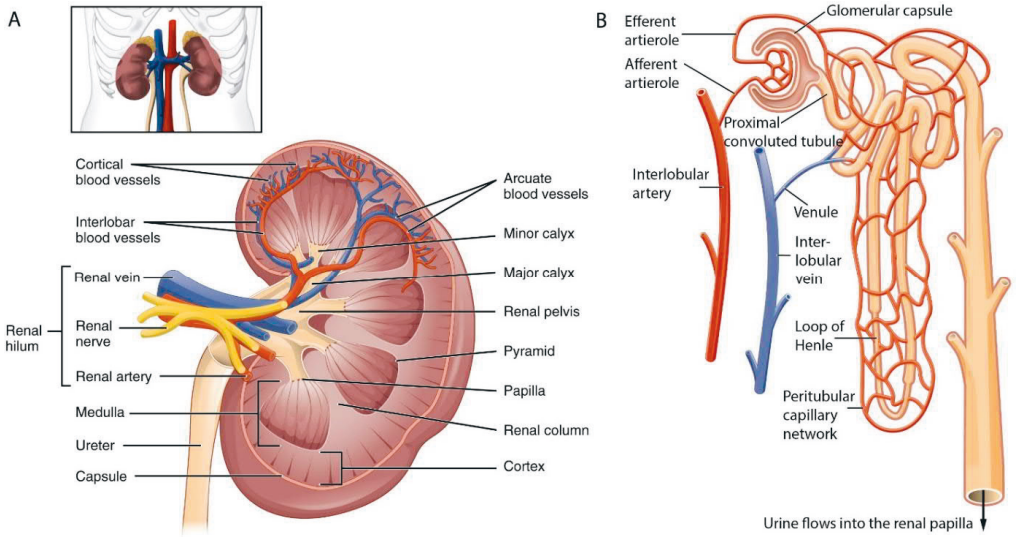

Figure 1: Structure of the kidney (A) and nephron (B), modified and reproduced from BC Open Textbooks according to CC BY 4.0

2.1.2. Glomerulus and glomerular filtration barrier

2.1.2.1. Structure and function of the glomerulus

Glomeruli are located in the kidney cortex and are the first part of the nephron, where the blood is filtered and primary urine produced. The glomerulus is enclosed by the Bowman's capsule. The afferent and efferent arterioles enter the Bowman's capsule by the vascular pole and form a tuft of capillaries wrapped by the glomerular basement membrane (GBM) and glomerular epithelial cells, also called podocytes. The succession of these layers forms the glomerular filtration barrier. All the layers contribute to the permselectivity of the glomerulus *via* both size and charge selection. Also, disruption of any layer of the glomerular filtration barrier can cause leakage of proteins into urine (10). After the ultrafiltration, the primary urine flows into the adjacent proximal tubule exiting the glomerulus *via* the urinary pole of Bowman's capsule (figure 2). Importantly, both afferent and efferent vessels are arteries, as the filtration in the glomerulus is based on static pressure. Renal hemodynamic function is controlled by hormonal and intrarenal autoregulatory mechanisms (11, 12). For example, the tubuloglomerular feedback influences the afferent arteriolar tone leading the control of intraglomerular pressure and glomerular filtration rate at the level of a single nephron (12).

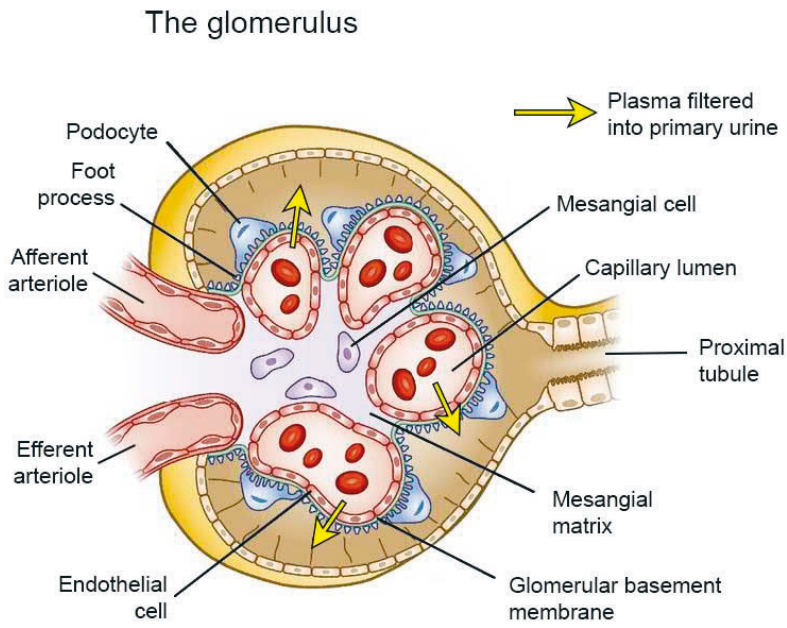

Figure 2: Structure of the glomerulus, modified from (13), © American Society of Nephrology, reproduced with permission

2.1.2.2. Glomerular endothelium

The endothelium lining the glomerular tuft is unique due to its fenestrations with pores of 50–100 nm. The luminal surface of the glomerular endothelium is coated with a glycocalyx layer that is negatively charged and participates in the charge-based selectivity of the glomerular filtration barrier (14).

2.1.2.3. Glomerular basement membrane

The core of the GBM is mainly composed of laminins and type IV collagen. These extracellular matrix components are secreted by both endothelial cells and podocytes, and are believed to participate in the size-selection of the permselectivity of the glomerulus (15). Genetic studies highlight the integral role of the extracellular matrix components for the maintenance of the filtration barrier. For example, mutations in collagen 4a give rise to Alport syndrome, a hereditary disease characterised by proteinuria and leading to end-stage renal disease (ESRD) (16).

2.1.2.4. Glomerular mesangial cells

Mesangial cells are mainly responsible for maintaining the structure of the glomeruli. They have a contractile activity that helps in regulating the intraglomerular pressure and bringing structural support to the capillary loops (8).

2.1.2.5. Glomerular visceral epithelial cells or podocytes

Glomerular visceral epithelial cells, also called podocytes, are the third component of the filtration barrier (figure 3). They are critical for the maintenance of ultrafiltration efficacy and prevention of the loss of proteins into the urine. Podocytes are terminally differentiated cells that cover the GBM on the urinary side. From their cell body, large primary processes originate and further divide into foot processes that connect with foot processes of adjacent podocytes, forming a typical cell-cell junction called slit diaphragm. The slit diaphragm relies on a number of proteins that are decisive for the maintenance of the structure of the podocyte foot processes. The slit diaphragm is a site of active endocytosis and exocytosis that are required for adaptation to the changing environment of the glomerulus (17). It is also a site of signalling and actin cytoskeleton regulation (18).

In many glomerular diseases, the elaborate structure of podocytes is lost. The retraction of podocyte foot processes into broader and simplified structures is called effacement. Also, in mature podocytes the typical organisation of the slit diaphragm, which is derived from tight junctions during the development, can be progressively lost. For example, in puromycin aminonucleoside nephrosis, the podocyte cell-cell junctions progressively lose the features of the mature slit diaphragm and are enriched in proteins typically found in tight junctions (19). Moreover, modern microscopy methods, such as serial block face-scanning electron microscopy and super resolution light microscopy, have unravelled that podocytes can protrude into the GBM and have an abnormally localised acto-myosin contractile apparatus in disease states (20, 21).

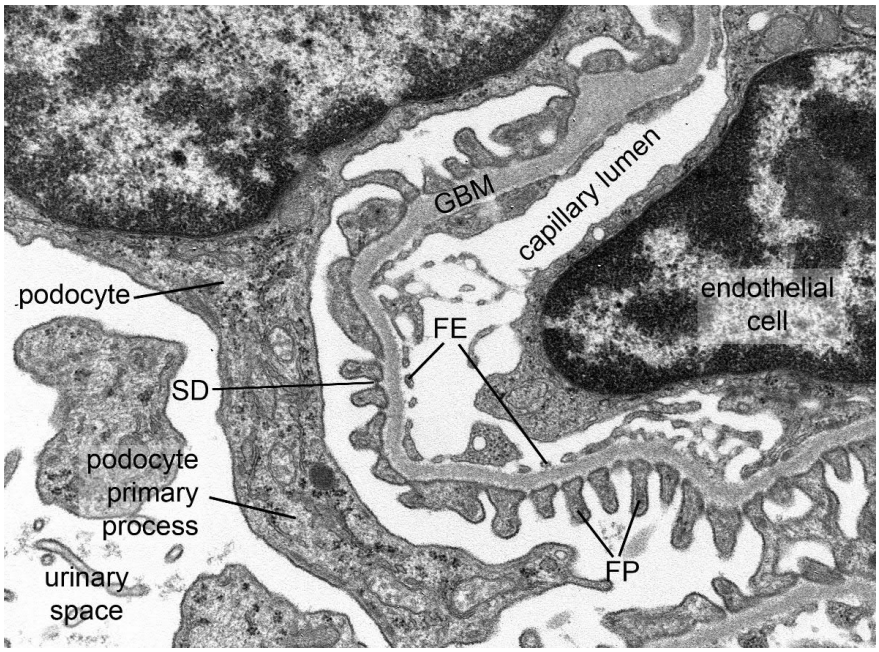

Figure 3: Electron microscopic view of the glomerular filtration barrier. FE: fenestrated endothelium, FP: podocyte foot process, GBM: glomerular basement membrane, SD: slit diaphragm

2.1.2.5.1. The slit diaphragm

The slit diaphragm is a highly regulated protein complex that joins adjacent podocytes (figure 4). Its structure relies on the presence of several proteins including integral slit diaphragm proteins, such as nephrin (22, 23) and Neph1 (24), proteins involved in the trafficking of the slit diaphragm proteins, such as podocin (25), and proteins anchoring the slit diaphragm to the actin cytoskeleton, such as CD2-associated protein (CD2AP) (26) and zonula occludens-1 (ZO-1) (27). Importantly, both nephrin and the related Neph1 harbour immunoglobulin (Ig) -like motifs, eight and five, respectively, and form cis- and trans-interactions (28-30). The interactions and the difference in the size of the Ig-like motif containing extracellular domains of nephrin and Neph1 result in the formation of a multilayered sieve, preventing proteins of the size of albumin and larger to leak into urine (30).

Mutations in *NPHS1* gene, encoding for nephrin, cause the congenital nephrotic syndrome (CNS) of the Finnish type in 1998 (22). The progression of the disease starts with proteinuria *in utero* and results in death of the patients during the two first years of life unless the patients receive a kidney transplant. In the original article, several mutations leading to CNS of the Finnish type were identified in the *NPHS1* gene and its product was named nephrin (22). Soon after this, podocin, encoded by the *NPHS2* gene, was identified as another gene responsible for early CNS (31). Strikingly, mutations in either *NPHS1* or *NPHS2* lead to defective trafficking of nephrin to the plasma membrane of podocytes (23, 25). These early studies pinpointed nephrin as a key molecule for the establishment of normal slit diaphragms in mature podocytes. To date, over 250 mutations in *NPHS1* and over 100 mutations in *NPHS2* have been reported to cause CNS (32, 33). The pivotal role for nephrin and podocin is confirmed by several total knockout models, which triggered early-onset and massive proteinuria (34-36). Also, inducible knockout models indicate that loss of nephrin or podocin, or of their interaction, in adulthood lead to slowly progressing focal segmental glomerulosclerosis (FSGS) and increased susceptibility of the mice to podocyte injury (37-40).

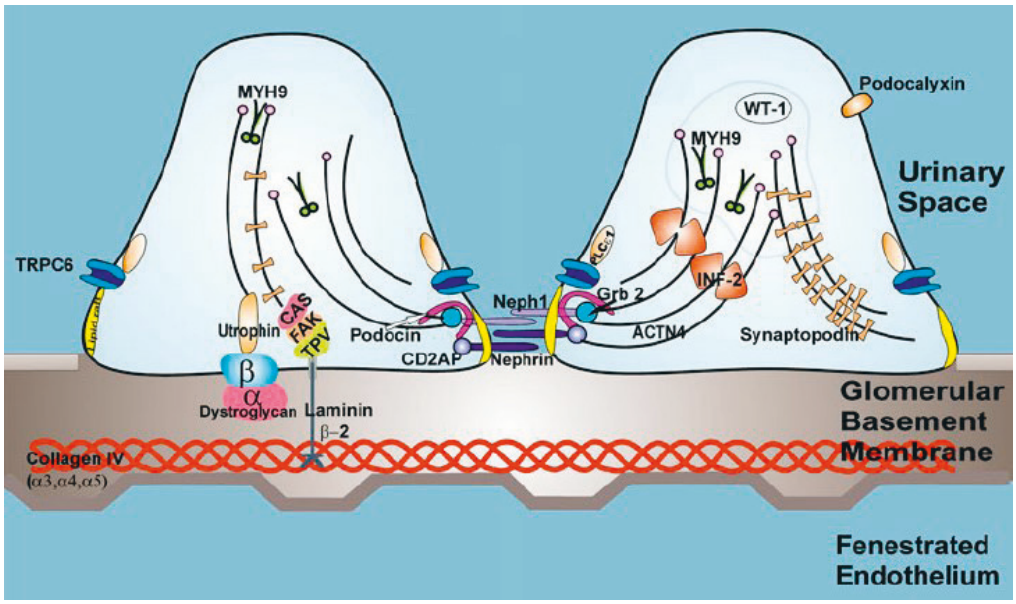

Figure 4: Proteins of podocyte foot processes and slit diaphragm, modified from (41), © Springer Nature, reproduced with permission

2.1.2.5.2. Actin cytoskeleton in podocytes

To support their highly organised structure, podocytes rely on the actin cytoskeleton. In the podocyte body and primary processes, filamentous actin (F-actin) is found together with intermediate filaments and microtubules (42), whereas in foot processes, only filaments of actin are present along the whole length of the processes (21, 43).

Many actin-associated proteins are shown to be essential to maintain podocyte function, including small GTPases that are significant regulators of actin dynamics in podocytes (44-47). In other cell types, they regulate migration, adhesion, division or polarity (48). In particular, the importance of RhoA, Rac1 and Cdc42 have been extensively studied in podocytes (44-47). Inducible podocyte-specific overexpression of constitutively active RhoA in adult mice results in reduced nephrin expression, foot process effacement and proteinuria, and this effect is reversed by removal of doxycycline (44, 45). Importantly, overexpression of a dominant negative RhoA also triggers reversible foot process effacement and proteinuria (44). Podocyte-specific knockout of Cdc42 results in congenital nephropathy and glomerular sclerosis, followed by death of the mice by the age of two weeks (46, 47). Podocyte-specific constitutive activation of Rac1 leads to podocyte effacement and loss, and albuminuria (49), whereas podocyte-specific depletion of Rac1 exacerbates chronic hypertension-induced albuminuria (46). However, depletion of Rac1 in podocytes prevents podocyte effacement induced by protamine sulphate perfusion (46). More conflicting is the fact that Rac1 depletion in podocytes has been found to protect against or aggravate streptozotocin (STZ)-induced DKD (50, 51). In addition, mutations in the Rho family dissociation inhibitor *ARHGDIA* lead to FSGS *via* defective signalling of Rho GTPases

(52, 53). The fact that both depletion and overexpression of constitutively active small GTPases can either trigger proteinuria or protect against renal injury supports the concept that a strict regulation of the actin cytoskeleton organisation is required for podocytes to adapt to their changing hemodynamic environment.

In addition to the small GTPases, mutations in *CD2AP* gene results in FSGS (54). Functionally, CD2AP anchors the slit diaphragm to the actin network by interacting with nephrin and F-actin, and deletion of CD2AP in mice results in death by the age of 6–7 weeks due to renal failure (26, 55, 56). Also, podocyte-specific knockout of the actin nucleator neuronal Wiskott-Aldrich syndrome protein (N-WASP) results in albuminuria by the age of 3 weeks (57). In line with this, deletion of Arp3, one of the components of the Arp2/3 complex that is an effector of N-WASP, prevents the establishment of arborized podocyte morphology *in vitro* and decreases the adaptability of podocytes to changing mechanical forces in the glomerulus *in vivo* (58). Actin filaments are anchored to the GBM *via* proteins such as integrin β4 and α3 and laminin subunit β2 (59-61). These proteins are shown to be necessary for the prevention of podocyte loss and for the adaptation to the changes in the glomerular environment (59-61). Also, absence of proteins crosslinking actin filaments or regulating actin polymerisation, such as α-actinin-4, cofilin-1, inverted formin 2 and myosin 1e, can induce the loss of podocyte function (62-65).

Finally, it has been demonstrated that post-translational modifications can regulate nephrin signalling and internalisation. For instance, the tyrosine kinase Fyn mediates the phosphorylation of human nephrin at Y1176/1193/1217, and that this leads to the recruitment of the adaptor protein Nck (66, 67). This, in turn, facilitates actin reorganisation with the appearance of actin comet tails (66). *In vivo*, the absence of Nck, both in development and adulthood, induces proteinuria (66, 68). This effect is apparently mediated by an improper activation of the N-WASP-Arp2/3 complex after Fyn-mediated phosphorylation of nephrin (69, 70).

2.1.2.5.3. Intracellular trafficking in podocytes

In addition to the strict regulation of the actin cytoskeleton organisation, also the regulation of intracellular trafficking in podocytes is decisive for the maintenance of podocytes and glomerular function. Recent findings have shifted the concept of the glomeruli and podocytes as stable structures towards a system requiring dynamic control to maintain its shape and function, and to cope with the stress that can result from haemostatic or metabolic pressure (71).

As previously mentioned, nephrin, together with other slit diaphragm components, needs to be localised at the plasma membrane to establish slit diaphragms necessary for the ultrastructure of podocytes. Mutations that prevented the trafficking of nephrin to the lipid rafts results in CNS of the Finnish type (22, 23, 25, 31). In addition to exocytosis, endocytosis has emerged as a mechanism necessary for podocyte survival by removing damaged molecules and regulating signalling from the slit diaphragm components (17, 18). Dynamins are large GTPases and master regulators of

endocytosis. They bind to and oligomerize around the neck of clathrin and nonclathrin forming vesicles, and are necessary for the pinching off of the vesicles from the plasma membrane (72). Dynamins appear to be necessary for maintaining the permselectivity of the glomerular filtration barrier as their proteolytic degradation has been found increased in proteinuric stages (73). Also, knocking out dynamins specifically in podocytes results in the foot process effacement, proteinuria and renal failure (74). Knockout of synaptojanin and endophilins, both functional interaction partners of dynamins, lead to a similar loss of podocyte and renal function (74). This is associated with defects in actin cytoskeleton organisation and nephrin endocytosis (74). In line with this, the class III phosphoinositide 3-kinase vacuolar protein sorting 34 (Vps34) maintains podocyte homeostasis by regulating the endocytic flux (75). Finally, constitutive activation of Notch signalling in podocytes induces the loss of proteins into urine in newborn mice due to reduced surface expression of nephrin (76). This effect is prevented by administration of a pharmacological inhibitor of dynamins (76).

The fact that knocking out CD2AP triggers postnatal death due to proteinuria (56), and that CD2AP haploinsufficiency increases glomerular susceptibility to injury (77), also argue for an essential role for endocytosis in the maintenance of the proper function of podocytes as the functional defects are mainly attributed to alterations in the degradation pathway (56, 77). Indeed, CD2AP colocalises with endophilin in the clathrin-mediated endocytosis site in podocytes (74). In addition, CD2AP associates with Rab4 and ADP-ribosylation factor-6 in other cell types (78, 79), both involved in endosomal trafficking (80, 81).

As controlled regulation of endocytosis is necessary for the maintenance of glomerular permselectivity (74), and the proper localisation of nephrin is necessary for podocyte function (23, 25), numerous studies have provided information on the mechanisms of nephrin trafficking (74, 82-89).

Nephrin endocytosis occurs *via* both clathrin-dependent endocytosis (CDE) and clathrin-independent endocytosis (CIE). The CDE has been proposed to be the rapid and constitutively active pathway that continuously internalises a small amount of nephrin, whereas the raft-mediated endocytosis would be slower, induced during the development or by damage, and internalises a larger amount of nephrin (84). The molecular switch that triggers the internalisation of nephrin and dictates the choice between these pathways appears to rely on nephrin phosphorylation. Phosphorylation of human nephrin at T1120/1125 allows the binding of β-arrestin2, which triggers the internalisation of nephrin by CDE (85, 86). The activation of Notch signalling in podocytes also results in a β-arrestin2- and dynamin-mediated CDE of nephrin (76). In addition, human nephrin phosphorylation at Y1176/1193/1217 is necessary for the maintenance of podocyte function as mutations of the three tyrosine residues to phenylalanines in mouse causes albuminuria during aging and increases susceptibility to glomerular damage (83). In some studies, the phosphorylation at Y1176/1193/1217 is necessary for the induced CIE of nephrin (84), whereas in others, reduced phosphorylation of Y1193 leads to nephrin internalisation *via* CDE (85).

Thus, it is known that podocytes can internalise molecules *via* several distinct pathways, and that the clathrin-dependent pathway is assumed to be predominant in

health (74, 84). Nevertheless, the mechanisms of nephrin internalisation are only partially elucidated, possibly due to the limitations of the podocyte models in cell culture and the difficulty of studying molecular mechanism *in vivo*.

2.1.3. Kidney tubules

The tubules are the second part of the nephron. They modify the primary urine passing from the glomerulus towards the collecting ducts. The first segment is the proximal tubule located in the cortex of the kidney and harbours highly endocytic cells with microvilli. It is first convoluted and then becomes straight towards the loop of Henle when descending deeper into the medulla of the kidney. Proximal tubules are a place of iso-osmotic reabsorption: a large proportion (~65%) of both water and ions that have passed the glomerular filtration barrier are reabsorbed. The filtrate enters the thin limb of the loop of Henle, first descending and then ascending, in which more reabsorption of water and exchange of ions occurs. Further, the distal tubule extends back next to the glomerulus and regulates the glomerular filtration rate based on the filtrate that goes through the distal tubule. The macula densa is the part of the tubule responsible for the connection with the glomerulus. It is a group of cells capable of sensing the content of the filtrate and secreting paracrine substances. As the tubule continues, more ions are reabsorbed, and the tubule descends again *via* the medulla towards the collecting ducts and ureter. During this process, the final concentration of urine is achieved by reabsorption of most of the water while the collecting tubules descend deeper into the kidney. Overall, most of the water and all the glucose that were filtered by the glomerulus are reabsorbed by the tubules in healthy conditions (8).

2.2. Diabetes mellitus

Diabetes mellitus is a chronic disease characterised by hyperglycaemia due to defective insulin production in the pancreas and the inefficacy of the insulin to trigger the cellular responses in the target tissues (also called insulin resistance). However, the mechanisms leading to the loss of control of the glycaemia are various and diabetes has been divided into several subtypes.

2.2.1. Types of diabetes

Type 1 diabetes mellitus (T1D) is also called insulin-dependent or juvenile diabetes. It is characterised by defective insulin production in the β-cells of the pancreas and requires insulin injections to normalise the glycaemia. The onset of T1D normally occurs in childhood or youth. T1D is an autoimmune disease with a strong genetic component. Overall, T1D represents less than 10% of all diabetes cases.

Type 2 diabetes mellitus (T2D) is a heterogeneous disease. It is characterised by insulin resistance, the incapacity of insulin-sensitive tissues, muscle, liver and adipose tissue, to respond to insulin and take up glucose from the blood. T2D is generally associated with obesity and the metabolic syndrome. In the early phases of T2D, insulin secretion

can be elevated as the body tries to compensate for the loss of metabolic control and for the loss of insulin sensitivity. However, as the duration of the disease increases, the compensatory overproduction of insulin attempting to normalise the glycaemia reaches a limit and the β-cells start to lose their capacity to produce insulin (90). The causes of T2D are not fully understood but a large part of the disease is attributed to lifestyle, and heritability traits can be found in up to 70% of the cases (91). As opposed to T1D, T2D is very common accounting for approximately 90% of diabetes cases. The usual onset of T2D is later in adulthood, although the prevalence of T2D in teenagers is increasingly observed (92).

Latent autoimmune diabetes in adults is another form of adult-onset diabetes which presents autoantibodies targeting the pancreatic islets but a slow loss of the β-cell function (93). Due to these features, this form of diabetes shows signs of both type 1 and type 2 diabetes. It accounts for about 10% of newly diagnosed diabetes amongst patients between 40 and 75 years (94).

Gestational diabetes is defined as a glucose tolerance disorder occurring during pregnancy. Gestational diabetes is usually asymptomatic but associates with an elevated risk of complications for the pregnancy and delivery as well as postnatal complications (95). Overall, gestational diabetes affects 13.2% of pregnant women according to a recent German study (96).

Finally, maturity onset diabetes of the young (MODY) is a hereditary monogenic form of diabetes. It is a rare disease (1–2% of all diabetes) and is mostly caused by a mutation in a gene of the hepatocyte nuclear factor family (HNF1-α, HNF1-β, or HFN4-α) (97).

This traditional classification of diabetes is largely accepted but the heterogeneity of diabetes, in particular the adult-onset diabetes, has recently incited Groop and co-workers to define a novel classification. The new classification of diabetes is based on clinical parameters measured at the time of diagnosis (98). These parameters include the age at the onset of diabetes, the level of glycated haemoglobin A1c, as well as estimates of beta-cell function and insulin resistance. The goal of the authors is to improve treatment strategies and to identify the risk of developing specific complications for different individuals. Interestingly, the cluster of people with severe insulin resistance presented an elevated risk for DKD (98).

2.2.2. Insulin: the key player in the development of diabetes

Insulin is a small peptide hormone produced by the β-cells in the islets of Langerhans of the pancreas. Upon postprandial increase of the glycaemia, insulin is secreted into the blood circulation from where it can reach muscles, liver and adipose tissue. In turn, these target tissues take up the glucose from the blood, increase their ATP production (99) and store the excess glucose as glycogen or lipids. Both defects in its production or its capacity to trigger a proper response in target tissues can lead to the development of diabetes.

2.2.2.1. Insulin signalling

Insulin is the major regulator of glycaemia as it signals to insulin-sensitive tissues and stimulates their uptake of glucose. Mainly studied in muscle, liver and adipose tissue, the canonical insulin signalling pathway is triggered by the binding of insulin to its receptor at the cell surface (figure 5). This leads to the autophosphorylation of the insulin receptor, recruitment of the insulin receptor substrate family proteins and their phosphorylation. The phosphatidylinositol 3' kinase (PI3K) is then recruited and activated, which turns the membranous PI(4,5)P2 into PI(3,4,5)P3. PI(3,4,5)P3 acts as a second messenger. It activates phosphoinositide-dependent kinase 1 (PDK1), which in turn activates both Akt, by phosphorylating T308, and mammalian Target Of Rapamycin (mTOR) C2, which further phosphorylates Akt at T473 (100, 101). The activation of Akt eventually leads to the translocation and fusion of GSVs to the plasma membrane, and entry of glucose into the cells (figure 5) (102). To turn off the signalling, Src-homology 2-domain-containing inositol 5'-phosphatase 2 (SHIP2) and phosphatase and tensin homolog negatively regulate this pathway by hydrolysing the 5' and 3' phosphate residues of the PI(3,4,5)P3, respectively (103).

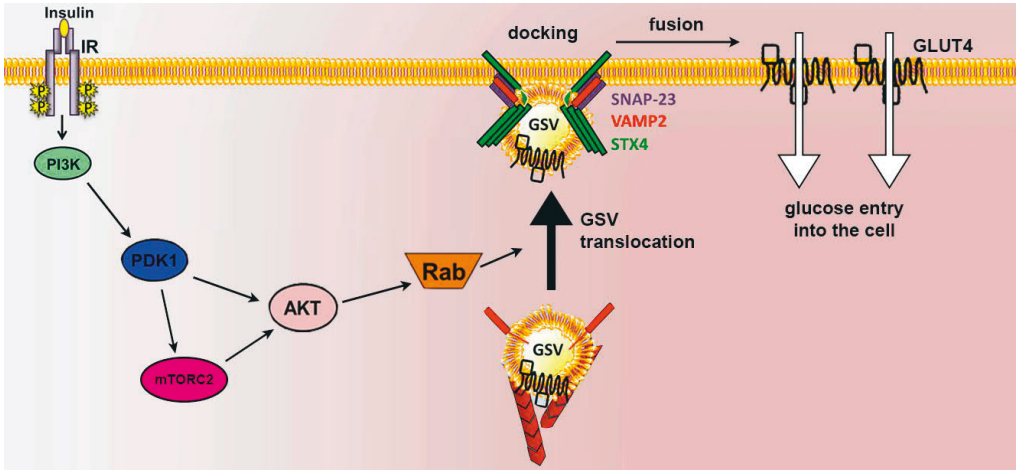

Figure 5: Insulin signalling pathway leading to the translocation of the GSVs to the plasma membrane and glucose entry in the cell, modified and reproduced from (104), © 2017 Tunduguru and Thurmond, CC BY 4.0

In addition to the main canonical pathway, insulin activates the mitogen-activated protein kinase pathway (105). In this case, Shc binds to the insulin receptor and signals *via* the SOS-RAS-ERK pathway to the nucleus which in turn regulates cell growth, differentiation and survival (105, 106).

2.2.2.2. Glucose transporter (GLUT) 4 trafficking

GLUT4 is synthesised in the endoplasmic reticulum, translocated to the trans-Golgi network and sorted into GSVs. In the basal condition, most of the GSVs locate in the perinuclear area of the cell (107). Upon insulin stimulation, the PI3K/Akt pathway inactivates Rab GTPase-activating proteins, which results in the activation of Rab

proteins, the major regulators of intracellular trafficking (102). This results in the translocation of 20–50% of the GSVs from the perinuclear region to the plasma membrane. Insulin stimulation eventually results in the fusion of GSVs with the plasma membrane and entry of glucose into the cell *via* GLUT4. The various stages of the insulin signalling pathway require finely controlled steps that include the trafficking of GSVs from the intracellular localisation towards the plasma membrane and the docking and fusion of the vesicles with the plasma membrane (figure 5) (108, 109).

The approaching GSVs relies on the actin and microtubule networks (104), and are tethered to the plasma membrane by the exocyst complex (110). The docking and fusion of the vesicles with the plasma membrane necessitates the SNARE (for soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) complex (110). The SNARE proteins are divided into v-SNAREs, expressed on the vesicles, and t-SNAREs, expressed on the receptor membrane, where the vesicle should be tethered. In podocytes, VAMP2 is the v-SNARE on GSVs and synaptosomal-associated protein 23 (SNAP23) is the t-SNARE at the plasma membrane (110).

When at the plasma membrane, GLUT4 can be internalised by both CDE, predominant in adipocytes stimulated by insulin, and CIE, predominant in unstimulated adipocytes (111). After endocytosis, the transporter can be recycled back to the plasma membrane if the insulin signalling is still active, or trafficked back to the GSVs in the perinuclear area (111).

2.2.2.3. Insulin resistance

Insulin resistance is a general term that is defined as the inability of the target tissue to respond adequately to the normal concentration of circulating insulin. In practice, it can be due to defects in the canonical insulin signalling pathway and/or in the trafficking of glucose transporters (112-114).

The metabolic alterations that lead to insulin resistance include, but are not limited to, uncontrolled glycaemia, and elevated levels of insulin, circulating free fatty acids (FFAs) and inflammatory cytokines (115). At the cellular level, the general metabolic changes transpose to generation of reactive oxygen species (ROS) and advanced glycosylation endproducts (AGEs), mitochondria-derived oxidative stress, endoplasmic reticulum stress and constitutive secretion of proinflamatory cytokines (116-120).

2.2.4. Complications of diabetes

Amongst individuals with diabetes, the fatalities are not directly due to the diabetic status but to the diabetic complications. The complications usually arise only after years of diabetes and uncontrolled glycaemia. The diabetic complications are divided into two major classes, macrovascular and microvascular complications. The macrovascular complications include coronary heart disease, strokes and other cerebrovascular diseases. They also comprise peripheral vascular disease that reduces the blood flow in the legs, which in turn prevents proper healing upon injury and may

lead to amputations (121). The microvascular complications include diabetic retinopathy, which can lead to the loss of vision, diabetic neuropathy, which damages the peripheral nerves, and DKD, which can eventually lead to ESRD (122).

2.3. Diabetic kidney disease

DKD is the renal complication of diabetes and the leading cause of ESRD. Approximately 40% of people with diabetes are likely to develop this microvascular complication (2). DKD is characterised by progressive albuminuria and declining glomerular filtration rate (GFR). Eventually, DKD may lead to ESRD, for which the only treatments are dialysis or kidney transplant. The first sign of DKD is persistent microalbuminuria, defined as albumin excretion rate (AER) of 30–300 mg/24 h. Further, the microalbuminuria can evolve to macroalbuminuria, defined as AER > 300 mg/24 h, or proteinuria defined as > 500 mg of protein in the urine per 24 h, which are considered as acquired DKD. Over the duration of diabetes, the GFR progressively erodes and a GFR < 60 mL/min/1.73 m^2 is also considered as a sign of overt DKD (123).

2.3.1. Typical course of diabetic kidney disease and clinical features

In T1D, microalbuminuria usually develops after 5–15 years of duration of diabetes. Microalbuminuria is considered as a risk factor for macroalbuminuria and the AER as a predictor of DKD (124-126). However, not all individuals with diabetes and microalbuminuria have the same risk to develop overt DKD. Up to 80% of people with T1D and microalbuminuria are likely to progress to macroalbuminuria within 6–14 years (124, 127). On the other hand, only 30–40% people with T2D and microalbuminuria are likely to progress to overt nephropathy within 10 years, possibly due to developments in the treatments and improved management of hyperglycaemia and hypertension (126, 128, 129). Finally, it is noteworthy that some individuals with diabetes and microalbuminuria may also regress to normoalbuminuria (130).

2.3.2. Risk factors for the progression of diabetic kidney disease

As mentioned before, the appearance of microalbuminuria in individuals with diabetes is a significant risk factor for DKD, especially in people with T1D or with insulin resistance (98, 124, 127). The AER can even be used as a prediction marker prior to the onset of microalbuminuria (131, 132).

The management of glycaemia is also a determinant for the risk of developing DKD. Myriad of studies have revealed an association between improved glycaemic control, by the means of intensified therapy, with insulin pumps or multiple daily injections of insulin, and a reduction of the appearance of microalbuminuria (3, 133-138). The improved quality of the glycaemic control results in a decrease in the glycated haemoglobin, the level of which correlates with the risk of both the appearance of

microalbuminuria and progression to proteinuria (139-141). However, this does not always prevent the progression to ESRD or reduce the all-cause mortality in individuals with diabetes (142, 143).

Hypertension is a pivotal factor involved in the progression from microalbuminuria to macroalbuminuria (136, 144, 145). Elevation in blood pressure normally occurs after the onset of microalbuminuria and is associated with the appearance of proteinuria (146, 147). In turn, hypertension increases the risk of acquired DKD (136, 144, 145).

Defects in the lipid metabolism are found early in individuals with T1D and albuminuria (148, 149), and are associated with increased risk of diabetic complications, including DKD (150, 151). Specifically, triglycerides and cholesterol predict the incidence of microalbuminuria and the progression to ESRD (152).

Another lifestyle-associated risk factor for DKD is smoking. Smoking is associated with an increased risk for microalbuminuria and acquired DKD (4, 153, 154). However, some variation of the association may be explained by the number of cigarettes smoked (145, 155).

2.3.3. Pathology of diabetic kidney disease

In DKD, the kidney presents defects in the glomerular and tubular compartments, as well as in the interstitial and vascular tissues.

2.3.3.1. Glomerular alterations

Amongst the histological findings in the glomeruli of people with DKD, the accumulation of extracellular matrix was detected as early as 1936 by Kimmelstiel and Wilson, who described hyalinous lesions typically in the shape of nodules (156). Consequently, the nodules are referred to as Kimmelstiel-Wilson nodules. More generally, the increase in the mesangial compartment, called mesangial expansion, includes the proliferation of mesangial cells and the accumulation of extracellular matrix proteins due to an increase in their secretion and decrease in their degradation (157-159). This may eventually lead to an enlargement in the glomerular area, the development of advanced diabetic glomerulosclerosis and reduction of the filtration surface area (157, 160, 161).

At the level of electron microscopy, the main finding in the diabetic glomeruli is the thickening and bulging of the GBM, observed already in 1959 (162, 163). The thickening of the GBM is an early feature of the progression of DKD as it can be detected as early as 1.5–2.5 years after the onset of T1D, and prior to the appearance of microalbuminuria (164, 165).

Electron microscopic examination of glomeruli can also reveal defects in podocytes. First, podocyte hypertrophy can be observed (166). As podocytes are terminally differentiated cells, it has been proposed that as a compensation for progressive loss of podocyte count, the remaining podocytes start to undergo hypertrophy to cover the exposed areas of the GBM. Hypertrophy occurs at the cellular level as well as at the level of foot processes that become wider. In individuals with DKD, or with diabetes

and elevated AER, the foot processes are effaced (166-168). Second, the slit diaphragm, the typical podocyte-podocyte junction, can be replaced by tight junctions, possibly as a consequence of the reduction of integral slit diaphragm proteins, such as nephrin (169).

In addition to the changes in the glomerular structure, the GFR varies during the course of the disease. In general, it is recognised that the GFR might increase at the onset of diabetes and prior to the development of microalbuminuria (170, 171). However, the decline in the estimated GFR (eGFR), calculated using the creatinine clearance method, is a clinical variable that should be monitored to evaluate the progression towards acquired DKD. If untreated, people with DKD have their eGFR lowered by 2–20 mL/min per 1.73 m^2 per year. However, the eGFR decline can be limited to 2–5 mL/min per 1.73 m^2 per year by controlling effectively the glycaemia and the blood pressure, blocking the renin-angiotensin aldosterone system and lowering the circulating cholesterol (172, 173).

Podocyte loss is another major mechanism leading to the deterioration of kidney function in both individuals with T1D or T2D, with or without DKD. Traditionally, it is thought to occur *via* detachment and apoptosis, but more recently, the presence of podocytes undergoing mitotic catastrophe in the urine has been reported (174-178). The number of podocytes remaining predicts the progression of DKD as it is estimated that the permselectivity is altered when 40% of podocytes or less are left (179, 180). Podocytes can be detected in the urine of both individuals with DKD and animal models of DKD (177, 181).

Finally, endothelial cells are altered in diabetes, as shown by elevation of the circulating von Willebrand factor and reduction of the endothelial glycocalyx (182-185). In the glomerulus, the proportion of the endothelium that is fenestrated is decreased in people with diabetes (178, 186).

2.3.3.2. Tubular changes

The proximal tubule cells are responsible for taking up glucose that is freely passing through the glomerular filtration barrier into the primary urine. At the onset of diabetes, the load of glucose that needs to be reabsorbed increases massively (187). This causes stress for the proximal tubule cells, which become unable to reabsorb it all. This, in turn, exposes distal tubule cells to glucotoxicity, as they are not normally in contact with high glucose concentrations. Consequently, it is in the distal tubule that the first pathological changes are observed, with the accumulation of glycogen (188, 189). In addition, albumin, which has pro-inflammatory and pro-fibrotic properties, starts to leak from the glomeruli and reaches the distal portion of the nephron (187, 190). Due to these changes in the primary urine content, interstitial fibrosis and tubular cell atrophy appear in DKD (191-193).

2.3.3.3. Vascular defects

Vascular hyalinosis of both arteries and afferent arterioles is observed in several renal vascular diseases. However, hyalinosis of the efferent arterioles of the glomerulus is a distinctive sign of DKD (194).

2.3.4. Mechanisms underlying the development of diabetic kidney disease

2.3.4.1. Molecular pathways deregulated in diabetic kidney disease

Diabetes is a complex disease, often occurring together with the metabolic syndrome, which implicates dysfunctions in many organs. This implies that the normal inter-organ crosstalks are disturbed, and thus, many circulating factors can contribute to the development of DKD. The circulating factors can in turn activate many interconnected intracellular pathways.

Elevation in the circulating blood glucose content, or hyperglycaemia, increases the *de novo* synthesis of diacylglycerol from glycolytic intermediates (195). In turn, diacylglycerol accumulation activates protein kinase C (PKC) family members, which will transduce deleterious signals *via* various effectors (196, 197). The downstream effectors include vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β) and nitric oxide (196, 197). PKC also activates the NADPH-oxidase, leading to the accumulation of ROS (196, 197). In addition, hyperglycaemia activates the renin-angiotensin aldosterone system, which controls the renal blood flow. Blockade of this system has proven effective in the treatment of DKD, in part by reducing ROS generation (198). Finally, the excess intracellular glucose leads to the synthesis of AGEs, which circulate in the blood stream of individuals with diabetes and activate their receptor, which in turn also participates in the generation of ROS (196, 197). Altogether, the accumulation of oxidative stress affects several intracellular pathways, including the p38 and ERK1/2 mitogen-activated protein kinases, Akt and nuclear factor-κβ (NF-κβ) pathways. As a result, these pathways lead to lipid peroxidation, DNA damage and inflammation (199).

Connective tissue growth factor and VEGF are also cytokines dysregulated in DKD that affect matrix accumulation and vascular permeability (196, 200).

2.3.4.2. Inflammation

As mentioned above, hyperglycaemia stimulates ROS accumulation, PKC activation and receptor of AGEs signalling (196, 197). In turn, all these factors stimulate inflammation partially through the NF-κβ pathway (201). It is known that people with diabetes have low-grade inflammation prior to the appearance of complications (202). The systemic inflammation, monitored by measuring C-reactive protein, further increases concomitantly with the AER in both T1D and T2D (202, 203). As the inflammation progresses, pro-inflammatory molecules, such as monocyte chemoattractant protein-1, interleukin-(IL)-1, IL-6, IL-18, and tumour necrosis factor-

alpha (TNF-α), accumulate in the circulation and in the tissues (204-209). The pro-inflammatory molecules then activate deleterious reprogramming in target tissues and cells, including podocytes, leading to their loss of function and increased AER (201, 209).

2.3.4.3. Hypoxia

Another mechanism driving the progression of DKD is hypoxia (210, 211). Renal hypoxia is attributed to increased oxygen consumption by mitochondria (212). However, complications of type 2 diabetes may result from a general pseudohypoxic state due to a NADH/NAD redox imbalance (213). Hypoxia is generally harmful for kidneys as it can induce nephropathy independently of hyperglycaemia, hypertension or oxidative stress (212). In the case of DKD, hypoxia has been observed prior to albuminuria in a rodent model of T1D (214). Interestingly, a dietary intervention in rats with T1D reduced the progression of renal disease, apparently by reducing hypoxia and oxidative stress (215).

2.3.4.4. Alterations of the glomerular cell crosstalk

In addition to the systemic changes described above, the crosstalk between glomerular cells normally mediated by cell-cell contacts and the release of cytokines is disturbed in DKD. Podocytes participate in the increased production of VEGF in DKD, and endothelial cell express its receptors VEGF-receptor 1 and 2 (216). This signalling is elevated in DKD and its inhibition appears to prevent proteinuria (216-218). Another important actor in the endothelial-podocyte dialogue is the endothelial nitric oxide synthase (eNOS). Deficiency in eNOS has been shown to worsen DKD in a murine model of diabetes (219). This is supported by the association of polymorphism in the eNOS gene (*NOS3*) and the risk of DKD (220). Other factors that could regulate the endothelial-podocyte crosstalk include endothelin and insulin-like growth factor, reviewed in (216).

Podocytes and mesangial cells secrete collagens and fibronectins and are responsible for the maintenance of the GBM (15, 221, 222). The production of the extracellular components in both podocytes and mesangial cells is regulated by TGF-β (221, 222). Importantly, TGF-β is upregulated in various glomerular diseases and in diabetes, in which it plays a role in the development of glomerular sclerosis (223-226). The overexpression of TGF-β in the glomeruli in diabetes is mediated by angiotensin II and AGEs (227, 228). The expression of TGF-β also stimulates ROS generation and the loss of podocytes by apoptosis (229, 230).

2.3.4.5. Mechanisms driving podocyte injury in diabetic kidney disease

In addition to the general mechanisms associated with diabetes and the progression to DKD, a few specific changes observed in podocytes are significant for this study.

2.3.4.5.1. Downregulation or mislocalisation of nephrin

As described in paragraphs 2.1.2.5.1. and 2.1.2.5.3., nephrin is critical for the podocyte function as its downregulation or mislocalisation lead to albuminuria (231-234). A decrease in the level of nephrin has been reported at both protein and mRNA levels in human DKD (231, 232). Loss of nephrin expression was also observed in STZ-induced DKD in mice (233). Interestingly, the amount of nephrin expression inversely correlates with the AER (232). In addition, nephrin is mislocalised in a model of DKD where mTORC1 signalling is constitutively activated (234). Moreover, inhibition of mTORC1 in db/db mice restores the normal localisation of nephrin (234). Importantly, PKC activity is known to be higher in diabetes (196). Specifically, the activity of PKCα is increased in the glomeruli as a consequence of hyperglycaemia (89). This in turn triggers the phosphorylation of human nephrin at T1120/1125 and accelerates the endocytosis of nephrin *via* a β-arrestin2-PICK1-nephrin complex, apparently *via* CDE (86, 89).

2.3.4.5.2. Actin cytoskeleton deregulation

In diabetes and many other podocytopathies, the normal actin cytoskeleton organisation is disrupted. In DKD, actin is misplaced from the centre of podocyte foot processes to the basal domain where it lines the GBM (235). Dephosphorylation of human nephrin at Y1193/1217 by SHP-1 is observed in hyperglycaemic conditions *in vitro* and in Akita mice (236, 237), leading to alterations in the regulation of the actin cytoskeleton *via* Nck (66). Also, hyperglycaemia and TGF-β induces a reduction in the expression level of SRGAP2a in DKD (238). Furthermore, SRGAP2a is a regulator of the small GTPases of the Rho family, and lowering of its expression inhibits podocyte motility (238). Overexpression of SRGAP2a in db/db mice attenuates the loss of podocytes (238). Palladin is an actin bundling protein found downregulated in human DKD (239). Its knockdown reduces F-actin and increases podocyte susceptibility to cytochalasin D-induced actin disruption (239). *In vivo*, podocyte-specific knockout of palladin increases the susceptibility of the mice to nephrotoxic serum-induced injury (239). Finally, hemicentin-1 is upregulated in human DKD (240). Hemicentin-1 is an extracellular matrix component whose expression in podocytes is regulated by high glucose and TGF-β. In podocytes, silencing hemicentin-1 partially prevents TGF-β induced actin cytoskeleton redistribution (240).

2.3.4.5.3. Defects in insulin signalling

As originally discovered in 2005, podocytes express the insulin receptor and are insulin-sensitive cells (241). Upon insulin stimulation, glucose transporters 1 and 4 are translocated the plasma membrane, increasing the glucose entry into podocytes (241). Moreover, the importance of the insulin signalling pathway was confirmed *in vivo* by a study showing that knocking out the insulin receptor specifically in podocytes in mice results in glomerular changes reminiscent of DKD, although the blood glucose levels of the mice remains normal (7). *In vitro*, depleting the insulin receptor substrate 2 in podocytes also results in insulin resistance (242).

Interestingly, nephrin is required for the full effect of insulin on podocytes, as it appears to help the docking of the v-SNARE VAMP2 to the plasma membrane, required for the final steps of GLUT4 translocation to the plasma membrane (243). Recently, our laboratory found that septin 7 inhibits glucose uptake in podocytes in both basal conditions and after insulin stimulation (244). Indeed, septin 7 prevents the interaction of the v-SNARE VAMP2 with nephrin at the plasma membrane (244). Also CD2AP, which links the slit diaphragm to the actin cytoskeleton (26, 55), is expressed on GSVs and regulates the trafficking of the insulin-responsive GSV pool in podocytes (245).

In DKD, several components required for efficient insulin signalling are altered in podocytes. Interestingly, loss of insulin receptor and subsequent insulin resistance is achieved by incubating podocytes in a culture medium mimicking diabetic conditions that contains high glucose, high insulin, TNF-α and IL-6 (246). Also, the activity of the PI3K/Akt pathway is reduced in the glomeruli of obese Zucker rats, and insulin resistance is observed in podocytes of db/db mice prior to the onset of albuminuria (247, 248). In line with this, the expression of SHIP2, a negative regulator of the insulin pathway, increases in the glomeruli of Zucker rats, prior to the development of albuminuria, suggesting that insulin resistance may be a driving factor for the loss of podocytes and decrease in the renal function (249). Moreover, nephrin and CD2AP, which are essential for podocytes and participate in the regulation of insulin sensitivity of podocytes (22, 56, 243, 245), are decreased in diabetic conditions (231, 232, 250). Recently, our laboratory demonstrated that metformin, a first line drug for the treatment of diabetes, has renoprotective effects (251). Mechanistically, metformin prevents podocyte loss by acting as an insulin sensitiser by inhibiting SHIP2, thereby restoring the activity of the PI3K/Akt pathway and inhibiting the endocytosis of GLUT4 (251).

In humans, the importance of the insulin signalling in podocytes is also supported by the novel subclassification of T2D proposed by Ahlqvist and colleagues that highlights the association of DKD specifically with the cluster of individuals with severe insulin resistance (98). In both T1D and T2D, insulin resistance associates with higher prevalence of DKD and correlates with the degree of albuminuria (252-254).

2.3.6. Animal models of diabetes and diabetic kidney disease

Myriad animal models for diabetes and DKD are used, but none are recognised to fully recapitulate the human disease. Some rodent models can be produced by injection of drugs, some are due to natural mutations and some have been generated by genetic engineering.

2.3.6.1. Induction of diabetes by streptozotocin

STZ can be injected intraperitoneally into mice and intravenously into rats, and it induces the destruction of the pancreatic β-cells, resulting in an insulin-deficient model of T1D (255, 256). Mice injected with STZ develop albuminuria and moderate mesangial expansion, depending on the strain (257). The administration of STZ has

also been performed in hypertensive rats, which developed albuminuria more rapidly than in rats without hypertension (258).

2.3.6.2. Naturally occurring diabetes in rodents

Leptin is a major hormone controlling satiety. It is produced by adipose tissue and small intestine and activates the leptin receptor in the hypothalamus, thereby inhibiting hunger (259). Several laboratory animal strains have developed single gene mutations in leptin itself or its receptor, resulting in the loss of satiety, subsequent hyperglycaemia and obesity. In addition, the animals develop T2D-like features and to some extent DKD (260). Below are described the most common examples of the animal models used for T2D research with defective leptin system.

The ob/ob ("obese/obese") mice, described in 1950, have a mutation in the leptin gene (261, 262). Due to the mutation, the mice are obese and insulin resistant. Originally, the renal phenotype was described as mild but the background of the mice contributes to the severity of the phenotype. Crossing of the ob/ob model with black and tan brachyuric mice leads to more severe insulin resistance and hyperglycaemia, resulting in more severe kidney phenotype with podocyte loss and glomerular lesions (263, 264).

A very similar model to ob/ob model is the db/db ("diabetes/diabetes") model. Db/db mice, originally described in 1966, harbours a mutation in the leptin receptor gene leading to an abnormal splicing of the receptor and defective leptin signalling (265-267). Db/db mice are widely used for T2D research and as for ob/ob mice, the severity of the disease depends on the genetic background (268). Db/db mice develop early-onset DKD with elevation of the AER, mesangial expansion and GBM thickening (269). Recently, db/db mice have been crossed with the DBA/2J strain. This results in increased AER and insulin resistance compared to the most commonly used db/db BLKS/J, but similar glomerular lesions (270).

The obese "fa/fa" ("fatty/fatty") Zucker rats are the equivalent of db/db mice, as their leptin receptor gene has a mutation (271, 272). The Zucker rat model is a widely accepted model for the study of obesity and diabetes but the renal lesions occur late and are rather mild (273, 274).

Zucker Diabetic Fatty (ZDF) rats are a substrain of the Zucker rats, first detected in 1977, but successfully isolated as late as 1990. ZDF rats develop obesity, insulin resistance, T2D, hyperlipidaemia, moderate hypertension and progressive renal injury (275). The renal findings are more severe than in the original Zucker rats and include albuminuria, glomerular sclerosis, mesangial expansion, macrophage infiltration and interstitial fibrosis (274, 276).

Some other rodent models of DKD present mutations leading to defects in insulin secretion or insulin resistance. The $Ins2^{+/C96Y}$ Akita mouse line is a strain with a spontaneous mutation in the insulin 2 gene, leading to cytotoxicity in the β-cells. As a result, the mice develop an insulin-dependent diabetes with hyperglycaemia and albuminuria. In this model, the severity of the disease also varies depending on the background (277). The Agouti and KK mouse lines have both been reported to have insulin resistance and have been bred to produce a mouse strain that has albuminuria

and glomerular changes (278-281). Finally, the Goto-Kakizaki rat model shows altered insulin sensitivity but the renal changes are mild and have a late onset (282-285).

2.3.6.3. Transgenic mouse models

In addition to the natural mutations, several mouse lines have been genetically engineered to develop diabetes. OVE26 mice overexpress calmodulin in pancreatic β-cells, making them hypoinsulinaemic and resembling T1D (286). Crossing db/db with mice with mice knocked out for eNOS (eNOS / db/db) is also used to worsen the renal phenotype of db/db mice (287). eNOS / db/db and OVE26 mice present high albuminuria and glomerular defects reminiscent of DKD (287, 288). In the eNOS / db/db model, the urea nitrogen is increased, which is rare in rodent models for diabetes (287).

2.4. PACSIN2

2.4.1. Identification and tissue distribution of PACSINs

Protein Kinase C and Casein Kinase substrate in neurons (PACSINs), or syndapins (for synaptic dynamin-associated protein), are mammalian homologs of the FAP52 chicken protein (289). The mRNA of PACSIN was originally found repressed after entorhinal cortex lesion in mouse (290). Plomann and coworkers further identified PACSIN2 and PACSIN3 sequences in both mouse and human (291, 292). In parallel, the rat homologs of PACSIN and PACSIN2, called syndapin 1 and 2, were sequenced, and two splice variants of syndapin 2 were described (293, 294). The distribution of PACSINs varies: PACSIN is neuron-specific (290, 293), PACSIN2 is ubiquitously expressed (292), and PACSIN3 is mainly expressed in skeletal muscle and heart (295).

2.4.2. Protein domains of PACSINs

Two protein domains are shared by PACSIN1-3. The first shared domain is the N-terminal F-BAR domain, for Fes-CIP4 homology Bin-Amphiphysin-Rvs161/167 (BAR) (292). The F-BAR domain is a conserved domain and a subclass of the larger BAR protein family (292). All BAR domains can bind to cellular membranes, as well as sense and induce their curvature. The curvature that the BAR domain applies to the membrane defines the subtype of the BAR domain (296, 297). F-BAR, N-BAR, PX-BAR and BAR-PH domains give a negative curvature to the membranes, meaning that the membrane is pulled towards the BAR domain protein. The F-BAR domain gives the shallowest curvature, followed by PX-BAR, N-BAR and finally the BAR-PH, which gives the most convex curvature. On the other hand, the I-BAR domain induces a positive deformation of the membrane, pushing the membrane away from the BAR domain protein (298). The BAR domains consist of α-helicoidal coiled coils, and their binding to cellular membranes rely on the presence of positively-charged residues in the concave surface of the banana-shaped BAR domain. These residues can interact with negatively charged lipids such as phosphatidylserine and phosphoinositides (296,

297). Importantly, BAR domains can oligomerize forming dimers and also larger and well-organised structures (298-300).

The second protein domain shared by all PACSIN isoforms is the C-terminal Src-homology 3 (SH3) domain. SH3 domain is a protein-protein interaction domain that can bind to a proline rich region of another protein. It enables the PACSINs to interact with a plethora of proteins involved in various cellular processes. Amongst the first proteins that were described to interact with the SH3 domains of PACSINs' were dynamin, synaptojanin, synapsin and N-WASP (291-294). Later, additional SH3 domain-mediated interactions of PACSIN proteins have been described with caveolin-1, Huntington protein, Cordon-bleu, mSon-Of-Sevenless, Rac1 as well as receptors such as CD95L, TRPV4 and TLR7/9 (301-308). These proteins are involved in endocytosis, both in CDE and CIE, actin cytoskeleton regulation and survival pathways. Another major function of the SH3 domain of PACSINs is the capacity to interact with the F-BAR domain and act in an autoinhibitory manner (299).

The central part of PACSINs is variable and flexible. Furthermore, PACSIN and PACSIN2 share 70% identity, and differ from PACSIN3 by the presence of multiple asparagine-proline-phenylalanine (NPF) motifs (291, 292). The NPF motifs of PACSIN are involved in N-methyl-D-aspartate receptor trafficking by interacting with the NR3A subunit of the receptor. Both PACSIN and PACSIN2 also associate, *via* their NPF motifs, with Eps15 homology-domain (EHD)-containing proteins, which are involved in endosomal recycling (299) (figure 6).

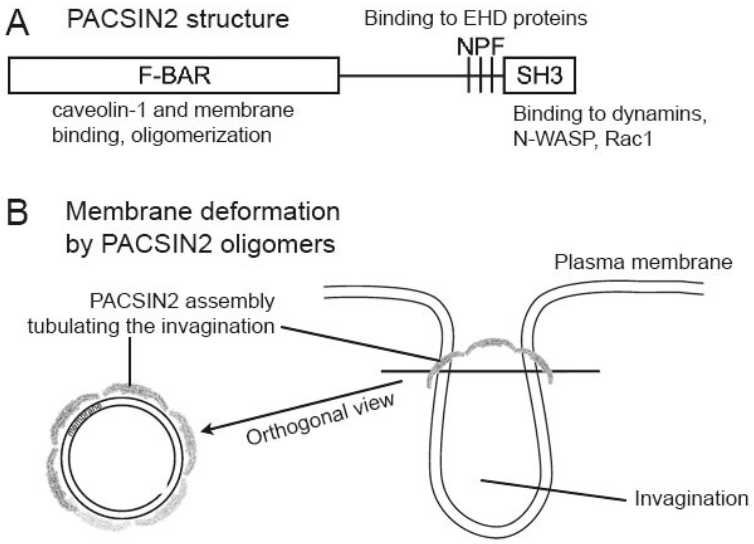

Figure 6: Structure (A) and induction of membrane curvature (B) by PACSIN2, modified from (300, 309), © Yosuke Senju and Shiro Suetsugu (CC BY-NC 3.0) and © John Wiley and Sons, reproduced with permission. F-BAR: Fes-CIP4 homology Bin-Amphiphysin-Rvs161/167 domain, NPF: asparagine-proline-phenylalanine motifs, SH3: Src-homology 3 domain, EHD protein: Eps15 homology-domain containing protein

2.4.3. Functions of PACSINs

Expressed in neurons, PACSIN regulates activity-bulk dependent endocytosis and synaptic vesicle formation and recycling, which in turn affects synaptic transmission and neuronal network activities (310-312). In line with this, seizures are observed in PACSIN knockout mice (310). In addition, PACSIN regulates actin cytoskeleton, neuronal morphogenesis, receptor signalling and cell migration. These aspects are described in detail in (299).

The best described function of PACSIN3 is the regulation of the trafficking of TRPV4, a Ca^{2+} permeable ion channel (307). Recently, a mutation in TRPV4 gene was suggested to affect its binding to PACSIN3 and to cause a congenital spinal muscular atrophy and arthrogryposis due to altered intracellular trafficking (313). In addition, PACSIN3 regulates GLUT1 trafficking in adipocytes (314).

Like PACSIN and PACSIN3, PACSIN2 has various functions, including regulation of endocytosis, actin cytoskeleton organisation, migration and receptor signalling as described in more detail below.

2.4.3.1. Role of PACSIN2 in endocytosis and intracellular trafficking

PACSIN2 was originally found to interact with dynamin, synapsin and synaptojanin, which are all involved in endocytic processes (292, 294). In the last 20 years, it has been shown that PACSIN2 participates in CIE, CDE, Golgi trafficking, and endosomal recycling (301, 315-320). First, it was reported that PACSIN2 overexpression inhibits the CDE pathway as shown by a decrease in transferrin uptake (292, 294). More recently, it has been reported that PACSIN2 is recruited to the clathrin-coated vesicles just before the pinching off of the clathrin-coated vesicles from the donor membrane (321). However, PACSIN2 function is more established in the caveolae-mediated endocytosis. In 2011, two studies revealed that knockdown of PACSIN2 using siRNA led to alteration in the caveolar architecture and prevented endocytosis *via* caveolae (301, 315). Immunogold labelling imaged by electron microscopy further confirmed the role of PACSIN2 in shaping caveolae (322, 323). In caveolae, PACSIN2 interacts with EHD2, cavin-1 and caveolin-1 (301, 315, 323). Recently, it was found that PACSIN2 phosphorylation at serine 313 (pS313-PACSIN2) regulates the lifespan of caveolae in response to osmotic chock, shear stress and loss of adherence by trypsinization (316). Upon phosphorylation, the membrane binding affinity of PACSIN2 is reduced, leading to dissociation of PACSIN2 from caveolae, recruitment of dynamin and internalisation of the caveolae (316). Interestingly, PKCα regulates the phosphorylation of this residue and PKCα is upregulated in the glomeruli in DKD (233, 316). In addition to endocytosis, PACSIN2 regulates endosomal recycling by interacting with EHD proteins (324). In this process, PACSIN2 appears to be necessary for nucleating tubular recycling endosomes and their fission from the donor membranes (319, 325). Finally, PACSIN2 participates in Golgi trafficking as it regulates the generation of carrier vesicles from the Trans-Golgi network (320, 326).

2.4.3.2. Regulation of the cytoskeleton by PACSINs

PACSIN2 regulates actin cytoskeleton and microtubule networks (292, 294, 327). Furthermore, PACSIN and PACSIN2 functions as molecular adaptors by recruiting actin cytoskeleton regulatory proteins to the plasma membrane (292, 294). For example, overexpression of full-length PACSIN2 induces filopodia and actin tails in an N-WASP-Arp2/3 dependent manner (292, 294). In line with this, overexpression of N-WASP blocks CDE only if its capacity to interact with PACSIN is maintained (328). More work on PACSIN demonstrated the role of PACSIN family members in the regulation of actin cytoskeleton. Targeting of Cordon-Bleu, an actin nucleation factor, to the plasma membrane is necessary for neuronal morphogenesis and depends on PACSIN function (303). PACSIN2 regulates actin cytoskeleton *via* Rac1, as PACSIN2 knockdown reduces cell spreading and migration, and decreases Rac1 activation (305). Interestingly, Rac1 activity also affects PACSIN2 localisation (305). Such regulation of actin cytoskeleton appears to rely on the phosphorylation of PACSIN at serine 358 by casein kinase 2 (residue equivalent to S399 in PACSIN2). Inhibition of the phosphorylation leads to a reduction in Rac1 activation and decreased spine formation in neurons (329). Mice knocked out for PACSIN2 also present decreased amounts of F-actin lining the brush border of the duodenum, which associated with a shorter actin core in the microvilli (330).

PACSINs also regulate the organisation of microtubules as they bind to unpolymerised forms of α- and γ-tubulin (327). Knocking down PACSIN2 with siRNA interference alters microtubule nucleation from centrosomes (327). However, the mechanisms whereby PACSIN2 regulates the microtubule network is very scarce and more studies are required to refine the role of PACSIN2 in this process.

2.4.3.3. Regulation of receptor signalling by PACSIN2

Early work on defining the role of PACSIN2 in endocytosis involved transferrin, revealing that PACSIN2 regulates transferrin receptor trafficking (292, 294). First, PACSIN2 inhibits transferrin receptor internalisation (292, 294). Second, when PACSIN2 is prevented to associate with EHD proteins, the transferrin receptor is not recycled back to the plasma membrane (324). PACSIN2 also regulates the expression of epidermal growth factor-receptor (EGF-R) at the plasma membrane, as PACSIN2 knockdown induces an accumulation of EGF-R at the cell surface. PACSIN2 knockdown also increases EGF-R signalling upon stimulation by epidermal growth factor (331). Intracellularly, PACSIN2 functions at the trans-Golgi network with OCRL1, a protein mutated in Lowe syndrome (320). Together, PACSIN2 and OCRL1 regulate the sorting of the mannose-6-phosphate receptor in trafficking intermediates (320). Finally, PACSIN2 has been suggested to participate in the sorting of the major histocompatibility complex I from the endosomal recycling compartments by functioning with MICAL-L1 (332). However, in this study, the authors did not directly test the involvement of PACSIN2 in the process; the assumption was based on their previous findings showing that PACSIN2 regulates sorting at the endosomal recycling compartment together with MICAL-L1 (319).

2.4.4. PACSIN2 in the kidney

The information about the function of PACSIN2 *in vivo* and in particular in the kidneys is very elusive. When I started my PhD project, no articles were published on the topic. However, two papers have been published during my studies. The first one describes PACSIN2 expression in the developing and adult kidneys (333). During development, PACSIN2 is found in distal and proximal tubules, in Bowman's capsule and sparsely in the glomerular tuft. In adult mice, the expression decreases in proximal tubule but increases in the glomeruli. Next, the authors linked PACSIN2 to repair processes: in an ischemia-reperfusion injury model in mice, PACSIN2 was found to be strongly upregulated in proximal tubules, where the damage was most significant. *In vitro*, the authors found that PACSIN2 regulates the length of primary cilia in tubular epithelial cells, and that PACSIN2 silencing alters the tubulogenic properties of the cells in 3D culture (333). In line with this, a recent study showed that PACSIN2 also regulates sensory cilia in the otic vesicle and olfactory placode of developing zebrafish (334). In a following study, Yao and colleagues provided evidence that PACSIN2 interacts with polycystin-1 (335). Interestingly, mutations in the polycystin-1 gene (*PDK1*) lead to polycystic kidney disease, characterised by the dilatation of the renal tubules. In this study, the authors found that PACSIN2, N-WASP and polycystin-1 colocalise in lamellipodia of migrating kidney tubular epithelial cells and regulate tubulogenesis *in vitro* (335). This is in line with previous literature revealing a role for PACSIN2 in actin cytoskeleton rearrangements (292, 294, 328).

2.4.5. PACSIN2 in diseases

In addition to a role in the kidney development and repair (333), PACSIN2 may have a role in HIV and *Clostrodium difficile* infections (336, 337). PACSIN2 knockdown prevents the HIV release and spreading (336). The mechanisms involve the components of the ESCORT complex, known to regulate the release of intraluminal vesicles from the multivesicular bodies. Also, the process depends on the capacity of PACSIN2 to interact with N-WASP and induce the reorganisation of the actin cytoskeleton (336). In addition, PACSIN2 is necessary for the internalisation of the two exotoxins produced by *Clostrodium difficile*. Toxins undergo endocytosis in a clathrin-independent but dynamin-dependent manner, in sites where PACSIN2 is expressed. The entry of the toxins into the target cells is also reduced by PACSIN2 knockdown (337).

Thiopurine is a drug used to treat acute lymphoblastic leukaemia (338). Some individuals receiving the treatment develop haematological and gastrointestinal complications. Polymorphism in *PACSIN2* gene is associated with both complications, although the link with the gastrointestinal complication is debated (339-342). In line with this, a recent genome wide association study linked a quantitative trait locus close to *PACSIN2* with the albumin/globulin ratio in pigs (343). Interestingly, lowering of the albumin/globulin ratio suggests renal defects, whereas its elevation implies gastrointestinal problems (343).

Finally, PACSIN2 has been associated with the cardiovascular system and platelet formation in a few studies. In foetal hearts, PACSIN2 knockout alters the electrophysiological properties of cardiomyocytes, and the alteration is due to changes in the expression of ion channels (344). Furthermore, PACSIN2 may be important for endothelial tissue maintenance (345). It is expressed in endothelial cell-cell junctions and appears to regulate the internalisation of VE-cadherin, important for the integrity of the endothelial monolayer (345). Finally, PACSIN2 functions in megakaryocytes where it regulates the tubulation activity necessary for the formation of novel platelets (346).

2.5. Septin 7

2.5.1. Septin family and structure of septin 7

Septins belong to the superfamily of P-loop NTPases (347). In addition to the GTP-binding domain, septins share a polybasic region, a septin unique domain and most septins also harbour coiled-coil domains. They can oligomerize to form highly ordered and regulated structures (figure 7) (348). The 13 septins expressed in humans are divided into subgroups (septin 2, 6, 7 and 9 subgroups). Interestingly, the septin 7 subgroup is only composed of septin 7 itself, whereas other subgroups comprise several septin isoforms. Typically, septins first assemble in heterohexameric or heterooctameric complexes, which always comprise two septin 7 molecules. The complexes can then further assemble into higher-order structures such as filaments, bundled filaments or rings (349, 350). Based on these properties, septins have been proposed as the fourth cytoskeletal component in the cells (348).

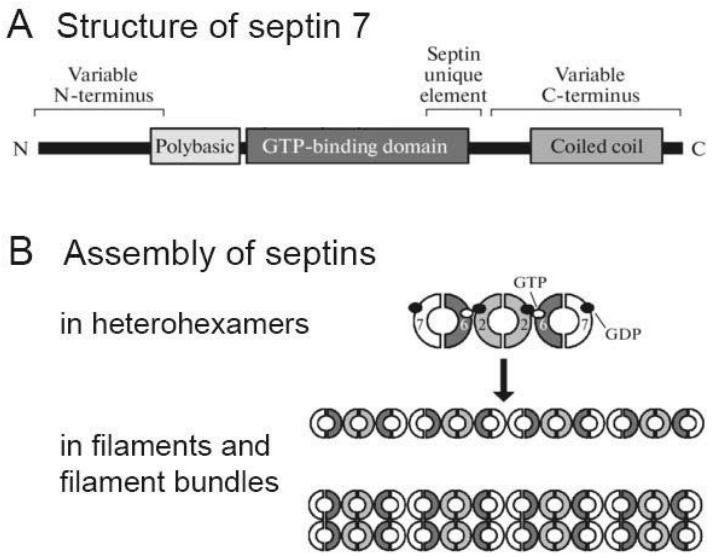

Figure 7: Structure of septin 7 (A) and assembly of septins into filaments and filament bundles (B), modified from (351). © Springer Nature, reproduced with permission

Of interest for our study, septins are also involved in intracellular trafficking events. When organised as filaments, septins can bind negatively charged membranes and induce a positive curvature (352). In particular, septin 7 is involved in the maturation process of the endosomes and regulates the formation of multivesicular bodies in a mechanism necessitating adaptor protein-3 and the ESCORT machinery (353). A recent paper shows that septins participate in the organisation of the endosomal compartment by controlling the remodelling of the actin cytoskeleton (354). Septins, including septin 7, can also oligomerize in a cage-like structure around *Shigella Flexneri* and promote its targeting to autophagosomes (355). Overall, it appears that septin 7 could be an important regulator of intracellular trafficking and the degradation pathway.

2.5.2. Function of septin 7 in the kidney

Septins function in various cellular processes, including shaping and compartmentalising the plasma membrane, regulating cilia formation and regulating cytokinesis (109, 348, 356). In the kidney, septin 7 is expressed in both tubules and glomeruli in mouse and zebrafish (244, 356). Septin 7 regulates fluid flow during development, and maintains the structure of podocyte foot processes and glomerular filtration in zebrafish larvae (356). In cultured mouse podocytes, septin 7 appears both as filaments, which colocalise partially with F-actin, and in a cytoplasmic vesicular pattern (244). Importantly, septin 7 interacts with CD2AP and nephrin (244), mutations in which lead to FSGS or CNS, respectively (22, 54). The filamentous organisation of septin 7 in podocytes relies on an intact actin cytoskeleton and the presence of CD2AP (244). Septin 7 also associates with VAMP2 (244), the v-SNARE involved in GSV trafficking, which was also found to interact with nephrin (243). Interestingly, disruption of septin 7 assemblies by siRNA silencing or treating podocytes with forchlorfenuron, which depolymerises septin filaments, increases glucose uptake in both basal and insulin-stimulated conditions, suggesting a potential role for septin 7 in the regulation of the trafficking of both GLUT1 and GLUT4 (244). Overall, septin 7 appears to have a role in kidney organogenesis *via* its function in cilia formation, and in podocytes, specifically in glucose transport, *via* mechanisms yet to be identified.

2.5.3. Other functions of septin 7 and its association with diseases

A vital role for septin 7 in cytokinesis is supported by the embryonic lethality observed in total septin 7 knockout mice, apparently due to mitotic failure (357). However, the requirement of septin 7 for the completion of cell division varies between cell lineages (357). The role of septin 7 in development is also supported by studies showing that knocking down septin 7 expression in zebrafish induces defects in the left-right asymmetry and in the differentiation of pancreatic endocrine cells (356, 358). Furthermore, silencing of septin 7 induces cardiac dysfunction apparently due to a disorganisation of cardiomyocyte myofibrils (359).

Overexpression of septin 7 in neurons of Drosophila induces flight defects, possibly by regulating Orai and inositol-1,4,5-trisphosphate receptor, suggesting a role for septin 7 in the regulation of calcium flux (360-362).

In the cancer field, septin 7 has been associated with glioma, papillary thyroid carcinoma, hepatocellular carcinoma, malignant mesothelioma and hereditary polyposis colorectal cancer (363-367). Interestingly, in these five cancers, septin 7 is described as a tumour suppressor as its overexpression inhibits cell proliferation and migration (364, 365), or negatively correlates with the malignancy of the tumour (366-368).

3. Aims of the study

Podocytes are critical for the maintenance of the renal function and are injured in DKD. Although numerous studies have aimed to clarify the pathological mechanisms underlying the development of DKD, the mechanisms leading to podocytes injury and the progression of the disease are not fully understood. This may partially be due to the lack of animal models recapitulating the whole spectrum of defects observed in the human DKD. In this thesis work, I aimed to characterise the renal phenotype of a new transgenic model of hyperglycaemia, as well as the involvement of PACSIN2 and septin 7 in the progression of DKD.

E1-DN mice are a novel transgenic mouse model of T1D (369). The mice express a mutated human EGF-R, known to act as a dominant negative for the murine EGF-R in pancreatic β-cells. The lowered EFG-R-driven signalling leads to reduced β-cells mass and insulin secretion, and, consequently, the homozygous mice develop hyperglycaemia by the age of two weeks (369). However, the renal phenotype of the E1-DN mice has not been characterised.

The literature pinpoints the importance of PACSIN2 in intracellular trafficking and in the organisation of the actin cytoskeleton. These processes are essential for the maintenance of podocyte function and are altered in DKD. Thus, I decided to evaluate the role of PACSIN2 in podocytes in the context of DKD, focusing on analysing its action on the actin cytoskeleton organisation and the trafficking of nephrin.

Deregulation of the insulin signalling pathway, and specifically, insulin resistance, is a risk factor for the development of DKD. Our laboratory has previously shown that septin 7 expression inversely correlates with glucose uptake in podocytes, possibly by affecting the docking and fusion of the GSVs with the plasma membrane. However, the detailed mechanisms were not well established. Thus, in my thesis project we aimed to decipher the role of septin 7 and its interaction partners in the process of GSV docking with the plasma membrane in podocytes.

The specific aims of the studies are the following:

1- To evaluate the renal phenotype of the E1-DN mice (study I)

2- To define the expression level of PACSIN2 and its phosphorylation at S313 in diabetes and diabetic kidney disease (studies II and III)

3- To characterise the role of PACSIN2 in the regulation of nephrin trafficking and actin cytoskeleton regulation (studies II and III)

4- To refine the role of septin 7 in the last steps of glucose transporter 4 storage vesicle trafficking in podocytes (study IV).

4. Materials and methods

4.1. Rodent models (studies I–IV)

The National Animal Experiment Board in Finland approved the protocols. The specific authorisation numbers can be found in the original articles. All animal experiments were performed according to the European Union guidelines.

E1-DN mice were generated previously (369) in FVB background. Shortly, a kinase-deficient EGF-R cDNA with a myc tag and a growth hormone polyA tail is expressed under a mouse Pdx1 promoter (369). Homozygous, heterozygous or their wildtype littermate male mice were used to examine their renal function in study I.

Lean (+/+ or fa+) or obese (fa/fa) male ZDF-Lepr^{fa}/Crt rats were obtained from Charles River Laboratories (Wilmington, MA, USA). Their metabolic parameters were measured and renal tissues collected as described below, and used for examining the localisation and expression levels of proteins (studies II and III).

Wildtype Sprague-Dawley, and lean (+/+ or fa/+) and obese (fa/fa) Zucker (Crl:ZUC-Lepr^{fa}, Charles River Laboratories) male rats were used to evaluate the expression, localisation and interaction of proteins (study IV). Detailed methods for the measurements of their blood glucose values, urinary albumin and creatinine measurements, tissue preparation and glomerular isolation are described in (249) and below.

4.2. Human material (studies II–IV)

The use of human material was approved by The Hospital District of Helsinki and Uusimaa Medical Ethics Committee. The specific authorisation numbers can be found in the original articles. All participants have signed a written consent for the use of the material.

Nonmalignant parts of the kidneys were collected after nephrectomies of individuals with renal cancer operated in the Helsinki and Uusimaa Hospital District. Glomerular isolation was performed as described below (studies II and III). The samples were divided based on the diabetes status of the people (study III).

Sera obtained from healthy controls, or individuals with T2D or T1D were collected by the FinnDiane Study Group (studies III and IV). The individuals were considered as having normoalbuminuria if the AER was normal (<30 mg/24 h), microalbuminuria with an AER of 30-300 mg/24 h, or macroalbuminuria if the AER was higher than 300 mg/24 h. The AER was measured in an accredited hospital laboratory (HUSLAB, Helsinki, Finland). For study III, sera from 20 individuals with T2D having normoalbuminuria (10) or microalbuminuria (10) were used to stimulate differentiated human podocytes. The FFA content of the sera was measured at the Biochemical Analysis Core for Experimental Research (BACER, University of Helsinki, Finland) using ADVIA 1650 (Siemens, Munich, Germany). For study IV, differentiated

human podocytes were incubated with sera collected from people with T2D, four individuals having normoalbuminuria and five individuals having macroalbuminuria. The fasting glucose and serum lipids were determined using a Hemocue device (Hemocue, Helsinki, Finland) or a Konelab analyser (Thermo Fisher Scientific, Waltham, MA, USA), respectively.

4.3. Metabolic measurements

4.3.1. Blood glucose and FFA measurements (studies I–III)

Blood glucose from E1-DN mice (study I) and ZDF rats (study II) was measured with OneTouch Ultra Glucometer (Lifescan, Milpitas, CA, USA) and Elite Glucometer (Bayer, Leverkusen, Germany), respectively. The FFA content of the sera from ZDF rats and people with T2D was measured at the BACER core using the ADVIA 1650 (Siemens).

4.3.2. Albumin excretion and estimation of the glomerular filtration rate (studies I and II)

E1-DN mice or ZDF rats were placed individually in metabolic cages and 24 h urine samples were collected. The volume of the collected urine was recorded and the concentration of albumin was determined using an ELISA kit (study I, CellTrend, Luckenwalde, Germany) or using ADVIA 1650 (Siemens) at the BACER core (study II). The total amount of albumin excreted was calculated as follows: $Excreted_{alb} = C_{alb} \times V_{urine\text{-}24h}$. The eGFR was calculated using the creatinine clearance method following the formula $C_{Cr} = (U_{Cr} \times V)/P_{Cr}$, ($C_{Cr}$: creatinine clearance in mL/min; U_{Cr}: urine creatinine in mg/mL; V: urine volume per min; P_{Cr}: plasma creatinine in mg/mL), and adjusted to the weight of the rat. The serum urea nitrogen and plasma creatinine were measured using the ADVIA 1650 (Siemens).

4.4. Cell Culture

4.4.1. Mouse podocytes (studies II and IV)

Wildtype mouse podocytes obtained from Dr Andrey Shaw (Genentech, South San Francisco, CA, USA) were used to produce a podocyte cell line stably overexpressing nephrin. For this, HEK293T cells were co-transfected with pLNCX2-nephrin (370) and the packaging pKAT2 vector with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Virus-containing medium was filtered to remove floating cells and used to infect podocytes. Cells transfected with nephrin were selected using geneticin (Merck, Darmstadt, Germany). The resulting new cell line is, hereafter, referred to as SNPs for "Shaw's nephrin overexpressing podocytes". These SNPs were maintained as the original mouse podocyte line (table 1).

4.4.2. Human podocytes (study III)

Human podocytes were maintained at 33°C in "permissive" conditions for proliferation or thermoswitched to 37°C for 7–14 days for differentiation. Cells were cultured in 5% CO_2 (table 1).

Table 1. Summary of cell lines and their culture conditions

Cell line	Culture medium	Culture temperature (°C)	Reference / supplier	Study
wildtype mouse podocytes and wildtype mouse podocytes stably overexpressing nephrin (SNPs)	DMEM containing 4500 mg/L glucose (Merck), supplemented with 2 mM ultraglutamine (Lonza, Basel, Switzerland), 10% inactivated foetal calf serum (Merck), 1X penicillin and streptomycin (Merck), and 10 U/mL interferon-γ (Merck)	33 in proliferation	wildtype: (371) stable nephrin overexpression: (372)	II, IV
human podocytes	RPMI 1640 containing 2000 mg/L glucose (Merck), supplemented with 2 mM ultraglutamine (Lonza), 10% foetal calf serum (Merck), 1X insulin-transferrin-selenium (Gibco), 1X penicillin and streptomycin (Merck)	33 in proliferation, 37 in differentiation	(373)	III, IV
HEK293T cells	DMEM containing 4500 mg/L glucose (Merck), supplemented with 2 mM ultraglutamine (Lonza), 10%	37 in proliferation	American Type Culture Collection, Manassas, VA, USA	IV

	inactivated foetal calf serum (Merck), 1X penicillin and streptomycin (Merck)			

4.5. Overexpression and knockdown strategies

4.5.1. Transient overexpression (studies II–IV)

Vectors were transfected into cells for transient overexpression using Lipofectamine2000 (Invitrogen) in antibiotic-free medium for 4 h. The cells were then cultivated in normal growth medium for 48 h prior to experiments. Table 2 lists the overexpression constructs used in studies II and IV, as well as their provenance.

Table 2. Constructs used for the overexpression studies

Gene (backbone vector)	**Species**	**Description**	**Vector backbone supplier / construct provider**	**Study**
eGFP-PACSIN2 (pCMV-eGFP-N1)	mouse	full length with eGFP	(374)	II
eGFP-N1 empty vector (pCMV-eGFP-N1)	N/A	control vector	Clontech, Mountain View, CA, USA	II
flag-PACSIN2 (pCMV-T2B)	mouse	full length with flag	Agilent Technologies (Technologies, Santa Clara, CA, USA)	II
flag-empty vector (pCMV-T2B)	N/A	control vector	Agilent Technologies	II
pacsin2-mCherry (modified from pCMV-eGFP-C1)	mouse	full length with mCherry	(301)	II

rabenosyn-5-eGFP (pCMV-eGFP-C1)	human	full length with eGFP	AddGene (Watertown, MA, USA)	II
Caveolin-1-DsRed-monomer (pDsRed-Monomer-N1)	mouse	full length with DsRed-monomer	(301)	II
clathrin light chain-eGFP	---	full length with eGFP	(301)	II
pCMV-myc vector	N/A	control vector	Clontech	III
myc-PACSIN2-wt	mouse	full length with myc	study III	III
myc-PACSIN2-S313E	mouse	phosphomimetic (phosphorylated) mutant with myc	study III	III
myc-PACSIN2-S313A	mouse	phosphomimetic (dephosphorylated) mutant with myc	study III	III
nephrin (pLNCX2)	rat	full length, wildtype	(370)	IV
pcDNA3.1	N/A	control vector	Invitrogen	IV
pcDNA3.1- Septin 7	human	full length, wildtype	subcloned from (375)	IV

4.5.2. Silencing gene expression using siRNA (studies II and IV)

Lipofectamine2000 (Invitrogen) was used to transfect wildtype mouse podocytes or SNPs with 150 nmol ON-TARGET plus SMARTpool mouse PACSIN2 (L-045093-01-0005), mouse NMIIA (L-040013-00-0005), siGENOME SMARTpool mouse septin 7 (M-042160-01-0005), or the siCONTROL Non-Targeting Pool#2 (D-001206-14-05) siRNAs (Dharmacon, Lafayette, CO, USA). The cells were used for experiments 48 h (PACSIN2, NMIIA) or 72 h (septin 7) post transfection.

4.6. Treating podocytes with high glucose, palmitate, PKC inhibitor or insulin (studies II–IV)

For high glucose treatment, SNPs were switched from 25 mM to 40 mM glucose (study II) and human podocytes from 11 mM to 30 mM glucose (study III). Mannitol was used as an osmotic control. Alternatively, human podocytes were stimulated with TNF-α at 10 ng/mL for 24 h. H_2O was used as a control (study III). Sodium palmitate (Merck) was conjugated to FFA-free bovine serum albumin (BSA, Merck) at a 3:1 molar ratio at 37°C for 1-2 h. SNPs (study II) or differentiated AB8/13 podocytes (study III) were treated with 50, 100 or 200 mM BSA-palmitate, as specified. BSA alone was used as control. When indicated, cells were treated with 200 μM bisindolylmaleimide I (Merck) in addition to palmitate stimulation (study III). Dimethyl sulfoxide was used as a vehicle control for bisindolylmaleimide I. Where indicated, podocytes were starved and stimulated with 20 nM insulin (NovoNordisk, Bagsværd, Denmark) for 15 min (study IV).

4.7. Lysis of cells and tissues, Western blotting

4.7.1. Isolation and lysis of glomeruli (studies II–IV)

Rat and human glomeruli were isolated from kidney cortices using graded sieving (376). The decreasing pore sizes of the sieves were 250/150/75 μm and 425/250/150 μm for rat and human glomeruli, respectively. The glomeruli, collected from the smallest-size sieve, were pelleted, resuspended in a small volume of phosphate buffer saline (PBS) and either directly lysed or snap frozen in liquid nitrogen for later use. For Western blotting, the glomeruli were lysed by sonication in 1% NP-40, 20 nM Hepes, 150 mM NaCl, pH=7.5 buffer (studies II-IV). For immunoprecipitation, the human glomeruli were lysed in Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, study II). All buffers were supplemented with 1x complete proteinase inhibitor cocktail (Roche, Basel, Switzerland), 50 mM sodium fluoride and 1mM sodium orthovanadate. The lysates were rotated for 30–60 min at 4°C, centrifuged at 16,000g for 15 min at 4°C, the soluble fractions were collected and the protein concentrations determined using Bradford assay (Merck) or the BCA kit (Pierce, Waltham, MA, USA).

4.7.2. Lysis of cells (studies II–IV)

Cells were lysed in Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Study II, immunoprecipications), NP-40 buffer (1% NP-40, 20 nM Hepes, 150 mM NaCl, pH 7.5) or RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, 150 mM NaCl, pH 8.0). All buffers were supplemented with 1x complete proteinase inhibitor cocktail (Roche), 50 mM sodium fluoride and 1mM sodium orthovanadate. The lysates were rotated for 15 min at 4°C and further processed as glomeruli, see 4.7.1. above.

4.7.3. Western blotting (studies II–IV)

Western blotting was performed as in (29). Shortly, lysates were mixed with Laemmli sample buffer containing 2.5% β-mercaptoethanol and boiled at 100°C for 10 min. Proteins were separated on 8–12% SDS-polyacrylamide gels and transferred to PVDF-FL membranes (Merck), which were blocked with Tris buffered saline-based Odyssey blocking buffer (LI-COR, Lincoln, NE, USA) or 5% skimmed milk in PBS and incubated with primary antibodies (table 3) for 1 h at RT or overnight (O/N) at 4°C. After final washes with PBS or Tris buffered saline, membranes were incubated with secondary antibodies (table 4) conjugated with either horse radish peroxidase, followed by chemoluminescent signal amplification (SuperSignal West Pico chemoluminescent substrate, ThermoFisher) for detection on Carestream Biomax films (Kodak, Merck; immunoprecipitations, study II), or with secondaries conjugated with fluorescent dyes (table 4) for detection using the Odyssey Infrared Imaging System scanner (LI-COR; analysis of the expression level of proteins and immunoprecipitations, studies II–IV). Blots were scanned and quantified using the Image Studio Software (LI-COR).

4.8. Histology, immunohistochemistry and immunofluorescence

4.8.1. Histological stainings (studies I, II and IV)

After dissection, mouse (study I), rat (study II) and human (study IV) kidney tissues were fixed in 10% formalin, dehydrated and embedded in paraffin. Five µm thick sections were deparaffinised and stained with haematoxylin-eosin or periodic acid-Schiff (PAS, study I) using standard procedures at the Department of Pathology, University of Helsinki, Finland. Slides were examined with a Nikon Eclipse 800 microscope (Nikon, Tokyo, Japan). PAS-stained E1-DN mouse kidneys were examined blinded of their genotype. Image-Pro Analyzer 6.0 (Media Cybernetics, Bethesda, MD, USA) was used to select in a colour-based manner the glomerular area that was PAS-positive. This area comprised mesangial cells, GBM and accumulating extracellular matrix, and was expressed as a percentage of the total glomerular area.

4.8.2. Immunohistochemistry (studies I, II and IV)

For immunohistochemistry, deparaffinised sections were subjected to antigen retrieval by boiling for 15 min in 10 mM acetic acid, pH 6. Samples were blocked in CAS-Block (Invitrogen) for 60 min at room temperature (RT), and incubated with primary antibodies (table 3) diluted in ChemMate (Agilent) O/N at 4°C. After washes in PBS, sections were incubated with secondary antibodies (table 4) and the development with aminoethyl carbazole was performed according to manufacturer's instructions (VectaShield Elite ABC kit, Vector laboratories, Burlingame, CA, USA). Sections were counter-stained with haematoxylin, mounted in Immunomount (Thermo Fisher Scientific) and examined with a Nikon Eclipse 800 microscope (Nikon). In study I, the

number of apoptotic cells, positive for cleaved caspase-3, per glomeruli were counted from 4–8 mice per group. E1-DN mouse kidney sections stained for Ki-67 were used to determine the proliferative index. The index was calculated as the percentage of Ki-67 positive tubular cells, calculated from 100 microscope fields (approximately 10,000 cells) per mouse (study I). For study II, PACSIN2 staining was quantified independently by two researchers blinded from the ZDF rat phenotype. The results included 15–24 glomeruli from 6–8 rats per group, scored from 0 (very weak staining) to 5 (very strong staining).

4.8.3. Immunofluorescence (studies I–III)

After dissection, mouse (study I) or rat (study II) kidney tissues were embedded and frozen in Tissue-Tek Optimum Cutting Temperature (OCT) compound (Sakura Finetek, Torrence, CA, USA). Kidney blocks were cut into 5 µm sections and fixed in 4% paraformaldehyde (PFA) in PBS for 30 min at RT. Alternatively, cells grown on coverslips were fixed with 2 or 4% PFA for 30 or 15 min at RT, respectively (studies II and IV). After rinsing with PBS, cells or sections were permeabilised using 0.1% Triton-X100 in PBS for 10 min at RT. Samples were washed with PBS, blocked with CAS-Block and incubated with primary antibodies (table 3) diluted in ChemMate (Agilent) for 1h at RT (cells) or O/N at 4°C (sections). After PBS washes, appropriate secondary antibodies (table 4) diluted in ChemMate (Agilent) were applied for 1h at RT. After final washes samples were mounted in Mowiol (Merck) or VectaShield (Vector Laboratories) with or without DAPI. Images were captured using wide field Zeiss Axioplan2 (Zeiss, Oberkochen, Germany), or Leica SP2, SP8 or SP8X confocal microscopes (Leica, Wetzlar, Germany). E1-DN mouse (study I) or ZDF rat (study II) kidney sections were stained for nephrin and examined by confocal microscopy. Images of the glomeruli were acquired using constant hardware and software setups for all animals. For study I, the intensity of nephrin staining was measured using Image J software from a stack of five consecutives images in the centre of the section, and divided by the glomerular area. For study II, the granularity of nephrin staining was determined independently by two researchers blinded from the phenotype. Randomised images of 6–8 rats per group, 8–10 glomeruli per rat, were scored from 0 (smooth) to 5 (granular). The granularity score was based on the overall granularity, the presence of punctate accumulations and the continuity of the nephrin staining along the GBM.

4.9. Electron microscopy methods

4.9.1. Structural electron microscopy (study I)

4.9.1.1. Sample preparation for structural electron microscopy

Cortical kidney cubes of 1x1x1 mm were fixed in 2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.2) for 2h at RT. Samples were postfixed in 1% osmium tetroxide for 2 h, stained en-block in 1% uranyl acetate in 10% ethanol for 1 h and embedded in LX-112 Epon (Ladd Research Industries, Willington,

VT, USA). Ultrathin sections were stained with uranyl acetate and led citrate, prior to examination with a Joel-1400 transmission microscope (Joel, Tokyo, Japan) equipped with an Olympus-SIS Morada CCD camera (Olympus Soft Imaging Solutions, Tokyo, Japan).

4.9.1.2. Glomerular mesangial fraction

As an alternative to PAS staining, the mesangial expansion in E1-DN mice was measured using electron microscopy. Glomeruli were examined by electron microscopy on slot-grids, allowing the quantification of the entire glomeruli. The mesangial fraction volume was defined as mesangial cells, extracellular matrix and the GBM, and estimated using a morphometric analysis based on the Delesse Principle as described (377). Shortly, a mosaic image representing a full glomerulus was produced from several individual images, a coarse grid was randomly applied to estimate the glomerular volume, and a thinner grid was applied to estimate the mesangial volume. The percentage of mesangial volume was calculated by dividing the number of times the crossings of the thin grid landed in the mesangial area by the number of times the crossings of the coarse grid landed on the glomerular area, the result was then multiplied by 100 and corrected by the ratio of grid sizes.

4.9.1.3. Foot process width

The foot process width was determined as described (378) using the ImageJ software. Shortly, the number of slit diaphragms was divided by the length of the GBM measured and multiplied by $\pi/4$.

4.9.1.4. GBM thickness

The thickness of the GBM was measured with the ImageJ software from random capillary loops. Areas with obvious bulging were excluded from the analysis to avoid overestimation.

4.9.2. Immunoelectron microscopy according to Tokuyazu's method (study IV)

Cortical kidney cubes of 1x1x1 mm were fixed with 4% paraformaldehyde in 0.1M PB, pH 7.2 containing 10% sucrose for 4–7 days at RT and infused with 2.3 M sucrose in PB O/N at 4°C. Blocks were frozen by immersion in liquid nitrogen. Ultrathin sections were quenched with 50 mM NH_4Cl in PB for 10 min at RT, blocked with 1% fish skin gelatin, 1% BSA in 50 mM NH_4Cl in PB for 10 min at RT, incubated with primary antibody (table 3) in 0.5% fish skin gelatin, 0.5% BSA in 50 mM NH_4Cl in PB for 60 min, washed multiple times with PBS and H_2O, and incubated with 10 nm gold-conjugated protein A (Department of Cell Biology, Utrecht School of Medicine, The Netherlands). After final washes, sections were stained with 2% neutral uranyl acetate for 10 min at RT and incubated in 2% methylcellulose, 2% uranyl acetate for 15 min on ice. Grids were then examined using a Joel-1400 transmission microscope (Joel) equipped with an Olympus-SIS Morada CCD camera (Olympus).

4.10. Interaction studies

4.10.1. Immunoprecipitation (studies II and IV)

Lysates from mouse podocytes, human podocytes, rat glomeruli or human glomeruli (200-500 μg of protein per reaction) were precleared with Protein A or G sepharose beads (Invitrogen) or with TrueBlot anti-mouse or anti-rabbit immunoprecipitation beads (Thermo Fisher Scientific), and incubated O/N at 4°C with primary IgGs (table 3) or purified IgG from rabbit or mouse (Invitrogen) as controls. The protein-antibody complexes were bound to the Protein A or G sepharose or TrueBlot immunoprecipitation beads for 2–3 h at 4°C and washed 3–5 times with the lysis buffer. Samples were boiled in Laemmli sample buffer containing 10% β-mercaptoethanol for 10 min. After separation in SDS-PAGE gels, expected interactions were characterised by Western blotting as described above. Alternatively, new interaction partners of PACSIN2 and septin 7 were identified by gel electrophoresis liquid chromatography-tandem mass spectrometry as described in (379). The data gathered was searched with in-house Mascot through ProteinPilot interface against the SwissProt database (Uniprot, http://uniprot.org/).

4.10.2. Proximity Ligation Assay (PLA) (studies II and IV)

Fixed cells were permeabilised, blocked and incubated with two primary antibodies (table 3) from different species as described for immunofluorescence. Samples were then processed for PLA according to manufacturer's instructions (Merck). Shortly, after washes, coverslips were incubated with mouse-minus and rabbit-plus secondary IgGs for 60 min at 37°C, washed in buffer A, incubated with the ligase mix for 30 min at 37°C, washed with buffer A, incubated in the polymerase mix for 100 min at 37°C, washed with buffer B and mounted on slides with Vectashield containing DAPI (Vector laboratories) or in the PLA mounting medium (Merck). Single IgGs were used as negative controls. The method enables the detection of antigens that are less than 30 nm apart. Images were captured with a Zeiss Axioplan2 microscope (Zeiss) and analysed with the Duolink Imaging Tool (Merck). For study II, signal per cell was defined as total signal per image divided by the number of nuclei in the image. In PLA experiments including Rabenosyn-5-eGFP or empty vector-eGFP overexpression, PLA signal was measured from individual cells overexpressing eGFP using manual segmentation of the images. For study IV, PLA signal of at least 100 cells per condition was measured with the automatic cell border definition by the Duolink Imaging Tool.

4.11. Nephrin trafficking assays (study II)

4.11.1. Structured illumination microscopy and total internal reflection fluorescence microscopy

Analysis of the colocalisation of PACSIN2 with nephrin at the plasma membrane and/or or in early endosomal tubules was performed using structured illumination microscopy (SIM) and live-cell total internal reflection fluorescence (TIRF)

microscopy. PACSIN2-eGFP was overexpressed and podocytes were incubated for 5–60 min in normal growth medium containing 516–647N IgG recognising the extracellular domain of nephrin (table 3). For SIM, cells were additionally washed and mounted in Vectashield (Vector Technologies). Both methods were performed on a Nikon Eclipse Ti-E microscope having either SIM or TIRF systems (Nikon).

4.11.2. On/In-Cell Western

SNPs were seeded on 96-well plates and transfected with flag-PACSIN2-wt or empty vector. On-Cell Western was performed to measure the amount of nephrin at the plasma membrane. Cells were transferred onto ice and incubated with 5-1-6 IgG for 15 min in 5% foetal bovine serum in PBS. After washes with cold PBS, cells were fixed with 4% PFA, blocked in 5% foetal bovine serum in PBS, and incubated with appropriate secondary antibody (table 4) and Draq5 (Thermo Fisher Scientific) for 20 min at RT. To define the turnover of nephrin, In-Cell Western was performed. For this, 5-1-6 IgG was added to the culture medium for the indicated times, cells were transferred onto ice for 15 min and processed as for On-Cell Western with an additional permeabilisation step with 0.1% Triton-X 100. For the recycling assay, cells were loaded with 5-1-6 IgG for 30 min and, subsequently, the plasma membrane-bound IgG was stripped by acid wash (1.15% acetic acid, 0.5M NaCl, 4 min on ice). Podocytes were returned to normal culture conditions for the specified times without 5-1-6 IgG and processed as for On-Cell Western. Draq5 was used to normalise the signal to the number of cells. The signal was measured using the Odyssey Infrared Scanning System (LI-COR). N = 33-36 wells from three individual experiments were used for statistical analysis.

4.12. Analysis of actin cytoskeleton and podocyte morphology by high-content screening (study III)

PACSIN2-wt or S313E/A cDNAs were transiently transfected into proliferating human podocytes and the coverslips were processed as described in 4.8.3. Specifically, cells were stained with CellMask Blue, Hoechst and anti-β-tubulin III IgG for segmentation. The cells were also stained with phalloidin-488 to stain F-actin and with an IgG targeting myc to select the cells that were positive for overexpressed wildtype or mutated PACSIN2. Imaging was carried out using the Opera Phenix HCS system (PerkinElmer, Waltham, MA, USA) with a 20x air objective, followed by processing with Cell Profiler 3.1.8 (380) to correct the illumination and segment the cells. Advanced Cell Classifier (381) was used to classify the cells based on the level of PACSIN2 overexpression or the appearance of the actin cytoskeleton and cell morphology. The cells were divided into three groups based on the intensity of myc staining: no, weak and high PACSIN2 overexpression. In terms of the actin cytoskeleton, the cells were divided into “normal” looking cells, having normal morphology with well-organised and clear actin stress fibres, or “altered” looking cells, with disorganised actin or changed overall cell morphology. A third class was used to

exclude rounded or dividing cells. The impact of myc-PACSIN2-wt/S313E/S313A overexpression was evaluated within individual coverslips by comparing the ratio of "normal" and "altered" cells amongst cells with high or no myc staining.

4.13. Glucose uptake (study IV)

SNPs were serum starved for 20 h, treated with or without insulin (20 nM) for 15 min in Kreps-Ringer PB (128 mM NaCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4$, 5.2 mM KCl, 10 mM $NaHPO_4$, pH = 7.4). 50 µmol/L (1µCi/mL) of tritium-labelled 2-deoxy-d-glucose (Perkin Elmer) was added for 5 min. Cells were washed with cold PBS and lysed with 1% Triton-X100 in PBS. Lysates were mixed with UltimaGold scintillation fluid (Perkin Elmer) and β-emissions were measured with a Wallac 1450 MicroBeta Trilux Liquid Scintillation Counter (Perkin Elmer).

4.14. Statistical analyses (studies I–IV)

For all studies, the differences between the groups were determined using Student's t-test if the n-number was equal or higher than 10 and if the samples were assumed to be normally distributed. In other cases, Mann-Whitney-U tests were used. $P<0.05$ was considered significant. P-values were calculated using SPSS PAWS Statistics (studies I and III, IBM, Armonk, NY, USA), Excel (studies I–IV, Microsoft, Redmond, WA, USA) or GraphPad v.6.02 (studies II and III, GraphPad Software, La Jolla, CA, USA).

4.15. Antibodies used for this work (studies I–IV)

Table 3. Primary antibodies used for the studies

Primary IgG	**Host**	**Supplier or reference**	**Study**
alpha-tubulin	mouse monoclonal	Merck	I–IV
beta-actin	mouse monoclonal	Merck	I–IV
beta-tubulin III	rabbit polyclonal	Merck	III
cathepsin D	rabbit polyclonal	Novus, Centennial, CO , USA	II
CD2AP	rabbit polyclonal	(55)	II
clathrin heavy chain	mouse monoclonal	Calbiochem, San Diego, CA, USA	II

cleaved caspase-3	rabbit polyclonal	Cell Signaling Technology, Danvers, MA, USA	I, IV
c-myc	mouse monoclonal	Merck	III
Ki-67	rabbit polyclonal	Bethyl Laboratories, Montgomery, TX, USA	I
nephrin	guinea pig polyclonal	Progen Biotechnik, Heidelberg, Germany	I, II, IV
nephrin #1034	rabbit polyclonal	(382)	IV
nephrin 5-1-6	mouse monoclonal	(383)	II, IV
nephrin 516-647N*	mouse monoclonal	(374)	II
nonmuscle myosin IIA heavy chain	rabbit polyclonal	Biomedical Technologies, Stoughton, MA, USA	IV
p62	guinea pig polyclonal	PROGEN	II
PACSIN2 "P2B"	rabbit polyclonal	Abgent, San Diego, CA, USA	II, III
PACSIN2 "P2P"	rabbit polyclonal	(292)	II, III
PACSIN2 phosphorylated at serine 313	rabbit polyclonal	(316)	III
phosphorylated myosin light chain 2 (Thr18/Ser19)	rabbit polyclonal	Cell Signaling Technology	IV
phosphorylated nonmuscle myosin IIA heavy chain (Ser 1943)	rabbit polyclonal	ECM Bioscience (Versailles, KY, USA)	IV
podocin	rabbit polyclonal	Merck	II
purified IgG used as control for IPs	mouse	Zymed (San Francisco, CA, USA)	IV

purified IgG used as control for IPs	rabbit	Zymed	II, IV
rab5	rabbit monoclonal	Cell Signaling Technology	II
rabenosyn-5	rabbit polyclonal	(384)	II
Septin 7 (C)	rabbit polyclonal	Immuno-Biological Laboratories, Gumma, Japan	IV
Septin 7 (H120)	rabbit polyclonal	Santa Cruz Biotechnology, Dallas, TX, USA	IV
Septin 7 (N12)	goat polyclonal	Santa Cruz	IV
SNAP23	rabbit polyclonal	Synaptic System, Goettingen, Germany	IV
SNAP23	mouse monoclonal	Merck	IV
syntaxin 4	rabbit polyclonal	Merck	IV
VAMP2	mouse monoclonal	Synaptic System	IV

*516-647N production (study II). We used NHS-ester Attodye 647N (Seigen, Germany) to fluorescently label the purified 5-1-6 IgG. Shortly, 400 µg of 5-1-6 IgG was incubated with 125 µM 647N-NHS-ester in 0.1M bicarbonate buffer in PBS for 60 min at RT. The free dye was removed by dialysis.

Table 4. Secondary antibodies and other fluorescent dyes used for the studies

Secondary IgG or fluorescent dye	**Host**	**Supplier or reference**	**Study**
10 nm gold-conjugated Protein A	N/A	Department of Cell Biology, Utrecht School of Medicine, The Netherlands	IV
Alexa Fluor 488-anti guinea pig	donkey polyclonal	Invitrogen	I, II
Alexa Fluor 488-anti mouse	donkey polyclonal	Invitrogen	II

Alexa Fluor 488-anti rabbit	donkey polyclonal	Invitrogen	IV
Alexa Fluor 555-anti mouse	donkey polyclonal	Invitrogen	I
Alexa Fluor 555-anti rabbit	donkey polyclonal	Invitrogen	I
Alexa Fluor 594-anti mouse	donkey polyclonal	Invitrogen	II
Alexa Fluor 594-anti rabbit	donkey polyclonal	Invitrogen	II
CellMask blue	N/A	Thermo Fisher Scientific	III
Hoechst 33342	N/A	Merck	II–IV
IRDye 680-anti guinea pig	donkey polyclonal	LI-COR	II–IV
IRDye 680-anti mouse	donkey polyclonal	LI-COR	II–IV
IRDye 800-anti mouse	goat polyclonal	LI-COR	II
IRDye 800-anti rabbit	donkey polyclonal	LI-COR	II–IV
Phalloidin-488	N/A	Thermo Fisher Scientific	III
TRITC-phalloidin	N/A	Invitrogen	IV
TrueBlot HRP-conjugated anti-mouse	rat monoclonal	Thermo Fisher Scientific	IV
TrueBlot HRP-conjugated anti-rabbit	mouse monoclonal	Thermo Fisher Scientific	II, IV

5. Results

5.1. E1-DN mice develop albuminuria, structural defects of the glomeruli and podocyte injury – study I

E1-DN mice express a truncated EGF-R in β-cells of pancreatic islets under the pancreatic duodenal homeobox-1 promoter (369). Due to impaired epidermal growth factor signalling, the postnatal growth of the β-cell mass is prevented, and the homozygous E1-DN mice have persistent hypoinsulinaemia (369). As a result, the homozygous mice present hyperglycaemia at the age of two weeks, which tends to decrease over time but remains higher than in their wildtype littermates (369). In (369), the authors focused on the description of the mouse line, the altered β-cell function and establishment of diabetes. Here, we evaluate the development of the diabetic kidney complication in this transgenic model of hyperglycaemia.

5.1.1. E1-DN mice are hyperglycaemic and albuminuric

As the onset of DKD occurs after weeks of hyperglycaemia, we used mice from 6 to 57 weeks of age to study the loss of kidney function in homozygous males. First, we confirmed that the homozygous E1-DN mice used for our study were hyperglycaemic at all time points analysed. We observed a tendency of improvement of the blood glucose over time (study I, figure 1a). Also, as in (369), heterozygous males had elevated blood glucose at 6 weeks but the level was normalised by the age of 40 weeks. Specifically, the blood glucose of homozygous male mice ranged from 30 mM at 6–7 weeks to 18 mM at 40 weeks. The heterozygous mice had 18 mM blood glucose at 6–7 weeks and 11 mM at 40 weeks, whereas the wildtype mice had 14 mM at 6–7 weeks and 10 mM at 40 weeks (study I, figure 1a). This confirms that E1-DN mice develop early-onset diabetes.

Next, we analysed the 24 h urine volume and its albumin content as a marker of the overall kidney function. In homozygous mice, the excreted urine volume in 24 h was elevated at all time points analysed compared to wildtype mice (although the statistical significance was not reached at all time points, study I, figure 1b). In heterozygous mice, however, the 24 h urine volume was elevated at weeks 6–7 but normalised by week 40 (study I, figure 1b), possibly reflecting the glycaemia (study I, figure 1a). The albumin content of the urine samples was measured and the AER was calculated for 24 h at 10 and 20 weeks. Homozygous E1-DN mice exhibited increased AER at both 10 and 20 weeks compared to both wildtype and heterozygous littermates (study I, figure 1c). At 20 weeks, the albuminuria was extremely high, in the range of milligrams per day. Strikingly, a strong variation was observed in the homozygous group in both blood glucose and AER, and we found a significant correlation between the blood glucose levels and the AER ($r = 0.71$, $p < 0.001$, study I figure 1d). Therefore, the elevation of the albumin concentration in the urine of homozygous and hyperglycaemic E1-DN mice justified more in-depth examination of the kidney phenotype.

5.1.2. Histological and structural changes in the kidneys of E1-DN mice

To characterise the morphological defects that could lead to the increased AER, we performed histological and proliferation analysis of the tubular compartment of the kidney, and evaluated the structural changes occurring in the glomeruli.

In the tubular compartment, haematoxylin-eosin staining revealed flattened epithelial cells and enlarged tubular lumens in homozygous mice with an AER superior to 1000 µg / 24h (study I, figure 1a, b). Using Ki-67 staining, we found that the proliferation of the tubular cells was increased from 0.18% to 0.70% in these mice, compared to wildtype littermates (study I, figure 3c, d). Such increase in proliferation is often considered as a sign of a repair process (385) and is in line with possible atrophy of tubular cells.

In the rest of this study, we focused on examining glomerular defects. In homozygous E1-DN mice, the mesangial area, defined as mesangial cells, extracellular matrix and the GBM, was found to be increased by 25% or 22% using PAS staining or evaluation by electron microscopy, respectively (study I, figure 2). In the two homozygous mice with the highest albuminuria, the accumulation of mesangial matrix was classified as focal, global nodular sclerosis (study I, figure 2d). Using electron microscopy, we also found that in homozygous E1-DN mice, the foot processes were wider (613 nm vs 423 nm, study I, figure 6e) and the GBM thicker (370 nm vs 258 nm, study I, figure 6d) than in their littermate controls. Occasional bulging of the GBM was also observed (study I, figure 6b). These morphological changes are reminiscent of the pathological findings of the human DKD.

5.1.3. E1-DN mice show increased podocyte apoptosis and decreased nephrin expression

To characterise the glomerular defects of the homozygous E1-DN mice in more detail, we measured the apoptosis rate in the glomeruli. We stained kidney sections of 20 and 57 weeks old E1-DN mice for cleaved caspase-3. At both age groups, increased glomerular apoptosis was found in homozygous mice compared to heterozygous and wildtype mice. At 20 weeks, the number of apoptotic cells per 10 glomeruli increased from 1.0 in heterozygous and wildtype mice to 6.5 in homozygous mice. At 57 weeks, the number of apoptotic cells raised to 4.4 in wildtype and heterozygous mice, and to 9.8 in homozygous mice (study I, figure 4a–c). Using double immunofluorescence with nephrin, we found that the glomerular cells positive for cleaved caspase-3 were podocytes (study I, figure 4d).

As nephrin has been shown to be decreased or mislocalised in DKD (231-234), and is necessary for normal glomerular function (22, 23), we compared its level of expression and localisation in homozygous E1-DN mice and wildtype littermates. The overall pattern of nephrin staining remained unchanged in homozygous E1-DN mice. However, the staining intensity was decreased in homozygous mice with albuminuria (study I, figure 5). As opposed to nephrin, the expression level of neither podocin nor ZO-1 was altered (not shown). Altogether, we show that in this model of

hyperglycaemia that shows signs typical of DKD, podocyte physiology is disturbed, resulting in reduced expression of nephrin and podocyte death.

To conclude, we found that homozygous and hyperglycaemic E1-DN mice develop serious renal complications that is reminiscent of human DKD with albuminuria, alterations in the glomerular ultrastructure, increased rate of podocyte apoptosis, and a reduction in nephrin expression.

5.2. PACSIN2 and its phosphorylation at serine 313 (S313) are increased in models of diabetic kidney disease but not in diabetes – studies II and III

PACSIN2 regulates the cytoskeleton and endocytosis in various cell types (292, 294, 301, 321). However, its role has not been examined in podocyte, a highly differentiated cell type that critically relies on the two above-mentioned cellular functions which are altered in DKD. Thus, we decided to investigate whether PACSIN2 could be involved in the pathological mechanisms underlying DKD.

5.2.1. Glomeruli of obese ZDF rats present elevated levels of total PACSIN2 and its phosphorylation at S313

To define whether PACSIN2 is involved in the development of DKD, we chose to use ZDF rats, a severe model of DKD with podocyte dysfunction (386). We measured the metabolic parameters from urine and serum of the rats at both 8 and 34 weeks, and collected the kidneys for further analyses. The detailed results of the metabolic analyses are found in study II, table 1. In brief, obese rats had elevated AER, urine albumin/creatinine ratio and urea nitrogen at both 8 and 34 weeks. Also, the eGFR was lowered in obese rats in both age groups.

Using immunohistochemistry, we showed that the total level of PACSIN2 is elevated in the glomeruli of obese rats at both 8 and 34 weeks (study II, figure 1a–c). Western blotting confirmed an increase in PACSIN2 levels in the glomeruli isolated from obese rats, but the increase was statistically significant at 34 weeks only (study II, figure 1d–f). In addition, immunohistochemical staining revealed that the signal for PACSIN2 tended to accumulate in cells harbouring the typical localisation of podocytes in the glomerular tuft (study II, figure 1a, arrows). The increased staining for PACSIN2 in podocytes was further confirmed by double immunofluorescence using nephrin as a podocyte marker (study II, figure 1g).

As previously shown (89), we observed that the expression of PKCα, responsible for the phosphorylation of PACSIN2 at S313 (316), is increased in the glomeruli isolated from 34-week-old obese ZDF rats (study III, figure 1e, f). We further found that pS313-PACSIN2 was increased in the glomeruli isolated from 34-week-old ZDF rats. Furthermore, the ratio of pS313-PACSIN2 to total PACSIN2 was also elevated at 34 weeks (study III, figure 1a–d). Thus, we demonstrate that PACSIN2 expression level is

abnormally elevated in podocytes of obese ZDF rats, and that PACSIN2 is also more phosphorylated at S313, probably as a result of enhanced PKCα activity.

5.2.2. Neither total PACSIN2 expression level nor its phosphorylation at S313 are changed in the glomeruli of individuals with T2D and normal kidney function

As the ZDF rats are a rodent model of severe diabetes and DKD, we aimed to examine whether the increase of total PACSIN2 expression level and its phosphorylation would occur already in diabetes or whether it is associated with the development of DKD. For this, we utilised human glomeruli isolated from nephrectomies due to cancer. The noncancerous part of the kidneys was used for the isolation of the glomeruli and the samples were divided into groups, depending whether the people have diabetes or not. No individuals had been diagnosed with diabetic kidney disease nor did they have known defects in their renal function. Notably, no significant difference was found in either the total PACSIN2 expression level or its phosphorylation at S313. A minor increase of the pS313/total PACSIN2 ratio was observed, but it did not reach statistical significance (study III, figure 2a–d). This result suggests that the total PACSIN2 expression level and its phosphorylation are not changed in the glomeruli of people with diabetes who do not yet have renal dysfunction.

5.2.3. Treating podocytes with sera from individuals with T2D and microalbuminuria increases PACSIN2 phosphorylation at S313

As PACSIN2 was found phosphorylated in the glomeruli of obese, albuminuric ZDF rats but not in the glomeruli of individuals with diabetes without clinical signs of kidney disease, we aimed to investigate whether the increase of pS313-PACSIN2 is associated with the loss of kidney function. For this, we treated differentiated human podocytes with sera obtained from people with T2D having normal AER or microalbuminuria. Interestingly, incubation of podocytes for 48 h with the sera from individuals with microalbuminuria increased the phosphorylation of PACSIN2 at S313 compared to incubation with sera from people with normal AER. The pS313/total PACSIN2 ratio was also slightly elevated but the difference was not significant as the total PACSIN2 level had also a clear trend of increase (study III, figure 2e–h). This, together with the analysis of glomeruli isolated from people with diabetes and normal kidney function, suggests that the changes in PACSIN2 expression and phosphorylation may not be associated with the onset of diabetes, but rather with the progression towards DKD.

5.2.4. High glucose and tumour necrosis factor-α treatments do not trigger the phosphorylation of PACSIN2 at S313

PKCα is known to phosphorylate PACSIN2 at S313 (316) and both PKCα and pS313-PACSIN2 are elevated in the glomeruli of obese ZDF rats (study III, figure 1). As PKCα activity is known to be regulated by high glucose treatment (86, 89), we hypothesized that the elevated glucose in the serum of obese ZDF rats could explain the increase of

PACSIN2 phosphorylation. To test the hypothesis, we differentiated human podocytes in high glucose conditions for 10–14 days and analysed PACSIN2 expression and its phosphorylation at S313 using Western blotting. Surprisingly, neither total nor pS313-PACSIN2 were changed by treating the podocytes with high glucose during the differentiation process (study III, supplemental figure 1). In addition to hyperglycaemia, circulating inflammatory factors are known to be increased in diabetes and DKD (201). Thus, we treated podocytes with TNF-α, known to stimulate inflammation *via* NF-κβ (387), for 24 h but this did not change total PACSIN2 expression or its phosphorylation at S313 (study III, supplemental figure 1). This suggests that the phosphorylation of PACSIN2 at S313 observed in the glomeruli of obese ZDF rats is triggered independently of hyperglycaemia or inflammation.

5.2.5. Palmitate treatment induces a time- and PKC-dependent phosphorylation of PACSIN2 at S313

Palmitate is the most abundant FFA in the circulation and is elevated in the serum from individuals with diabetes (388, 389). Thus, we treated differentiated human podocytes with palmitate bound to BSA for 4 min, 3 h or 24 h and measured the expression level and the phosphorylation of PACSIN2 at S313. These treatments were not sufficient to change the expression level of PACSIN2, but pS313-PACSIN2 and pS313/total PACSIN2 ratio were significantly elevated at both 4 min and 24 h (study III, figure 3a–f). Inhibition of classical PKCs with bisindolylmaleimide I suggested that the palmitate-induced phosphorylation of PACSIN2 was due to an increase of PKC activity *in vitro* (study III, figure 3g–j).

Next, we measured the FFA content of the sera from the ZDF rats and the people with T2D used in study III, figure 2e–h. In ZDF rats, the serum FFA concentration was higher in the obese than in the lean group at 34 weeks (study III, figure 4a). FFAs also tended to be more abundant in the sera from individuals with T2D and microalbuminuria compared to the ones having normal AER, but the difference did not reach significance (study III, figure 4b). Interestingly, we found a positive correlation between the phosphorylation of PACSIN2 at S313 in the glomeruli of ZDF rats and the FFA content of their sera ($r = 0.569$, $p < 0.05$, figure 4c). In the sera from individuals with T2D, a similar positive correlation was found but it did not reach significance ($r = 0.40$, $p < 0.10$, figure 4d).

Altogether, our results support the hypothesis that in DKD, PACSIN2 is phosphorylated at S313 in response to an elevation in the FFA content in the blood, and that this occurs *via* activation of PKC.

5.3. PACSIN2 regulates nephrin trafficking and actin cytoskeleton in podocytes – studies II and III

Nephrin is essential for kidney function and is mislocalised in various glomerulopathies including DKD (22, 23, 231-234). The proper regulation of the actin

cytoskeleton organisation is also necessary for podocyte function (5). As PACSIN2 regulates intracellular trafficking and endocytosis (292, 294, 301, 315, 316, 318-320) and actin cytoskeleton organisation (292, 294) in other cell types, we hypothesized that the aberrant increase of total PACSIN2 or its phosphorylation could alter these processes in podocytes in DKD.

5.3.1. Nephrin localisation is altered in the glomeruli of obese ZDF rats

In the glomeruli of obese ZDF rats, in which we found an elevated level of PACSIN2 expression, the total level of nephrin did not vary at either 8 or 34 weeks (study II, figure 2a–c), but its localisation was affected. In both age groups, the staining of nephrin appeared to change from a smooth lining of the capillary loops (study II, figure2d, left panels) to a more granular, dotty pattern (arrowheads, study II, figure 2d) with occasional aggregates of nephrin (arrows, study II, figure 2d). To quantify this, two researchers blinded of the phenotype scored the overall granularity in randomised images. At 8 weeks, a clear trend of increase in the granularity of nephrin was observed but the change in the granularity became significant at 34 weeks only (study II, figure 2e, f).

We aimed to define the nature of the structures containing nephrin aggregates, but, unfortunately, our attempt was not fruitful. Nephrin accumulations colocalised regularly with the other slit diaphragm proteins podocin and CD2AP, and were occasionally positive for clathrin. However, the aggregates were negative for rab5, cathepsin D or p62, markers of early endosomes, lysosomes and autophagic cargo, respectively (study II, supplemental figure 1). Nevertheless, our data show that nephrin displays an aberrant localisation in the glomeruli of obese ZDF rats having elevated PACSIN2 levels and defective kidney function.

5.3.2. Nephrin and PACSIN2 are internalised together and colocalise in various intracellular compartments in cultured podocytes

To define whether PACSIN2 plays a role in the trafficking of nephrin, we continued with *in vitro* studies and utilised a model of conditionally immortalized podocytes in which we stably overexpressed nephrin, hereafter called SNPs (characterised in study IV). Indeed, endogenous nephrin expression was lost in our podocyte cell lines, as is the case in most immortalized podocyte lines. Western blotting confirmed stable overexpression of nephrin in SNPs, and surface labelling of nephrin confirmed that nephrin is properly inserted at the plasma membrane (study IV, figure 1g–i). Next, confocal microscopy revealed that PACSIN2 and nephrin colocalise both at the plasma membrane and in intracellular compartments in SNPs (study II, figure 3a). PLA also confirmed that nephrin and PACSIN2 are separated by less than 30 nm (study II, figure 3c).

Next, we used the 5-1-6 IgG recognising the extracellular domain of nephrin and labelled it with the Attodye 647N (hereafter referred to as 516-647N). With this

fluorescently labelled IgG, we performed surface labelling followed by a 5 min chase in SNPs overexpressing PACSIN2-eGFP. Analysis with super resolution microscopy confirmed colocalisation of PACSIN2 and nephrin at the plasma membrane and in early endocytic tubules (study II, figure 3b).

Functionally, we examined the internalisation of nephrin by TIRF microscopy in SNPs overexpressing PACSIN2-eGFP. This method allows the visualization of molecular events within approximately 100 nm of the coverglass, and thus, it is used in molecular biology to investigate trafficking occurring at the plasma membrane. It was revealed with TIRF microscopy that nephrin, labelled with 516-647N, was internalised at various membrane regions, including large and immobile sites positive for PACSIN2, smaller and mobile areas also positive for PACSIN2 and areas devoid of PACSIN2 (study II, figure 4a, supplemental video 1). TIRF microscopy of SNPs overexpressing caveolin-1-DsRed-monomer also indicated that 516-647N-labelled nephrin or PACSIN2-mCherry were present in caveolae. Likewise, overexpression of clathrin light chain-eGFP followed by TIRF microscopy indicated that 516-647N-labelled nephrin or PACSIN2-mCherry are present in clathrin pits in SNPs (study II, supplemental figure 2). These results suggest that PACSIN2 regulates nephrin internalisation, occurring in both clathrin and caveolin-1 positive structures, as well as its intracellular trafficking.

5.3.3. PACSIN2 decreases the presence of nephrin at the plasma membrane but increases its turnover

To characterise how PACSIN2 regulates nephrin trafficking in a quantitative manner, we performed On-Cell and In-Cell Western experiments. First, surface labelling of nephrin with the 516-647N IgG without permeabilisation (On-Cell Western) showed that overexpression or knockdown of PACSIN2 decreases or increases nephrin insertion at the plasma membrane, respectively (study II, figure 4b, supplemental figure 3e).

The reduction of nephrin at the plasma membrane in PACSIN2 overexpressing SNPs could be due either to a decrease in the export of nephrin to the plasma membrane, or to an increase of its internalisation from the plasma membrane. To distinguish between these scenarios, we performed an In-Cell Western coupled with an incubation of the cells with the 5-1-6 IgG. Specifically, we incubated SNPs for 0 or 60 min with the 5-1-6 IgG followed by surface labelling to stain all nephrin molecules that have been present at the plasma membrane during this time, including the recently exported ones. The cells were further processed as for an On-Cell Western with an additional permeabilisation step. This approach revealed that after 60 min, a similar amount of nephrin molecules have been present at the plasma membrane in flag-PACSIN2 overexpressing cells (study II, figure 4c). This rules out the possibility that PACSIN2 overexpression prevents the export of nephrin to the plasma membrane. Moreover, when normalising the total nephrin stained after 60 min to the amount of nephrin present at the plasma membrane at a certain time, represented by t_0, we measured that the turnover of nephrin, represented by the fold increase between t_0 and t_{60}, is increased by flag-PACSIN2 overexpression (study II, figure 4d).

The concept of turnover of nephrin at the plasma membrane in the range of minutes to hours is supported by a recycling assay demonstrating that a significant amount of surface-labelled nephrin is returned to the plasma membrane between 15 and 120 min (study II, supplemental figure 3g, h).

Altogether, our On- and In-Cell Western results support the idea that total PACSIN2 overexpression accelerates the turnover of nephrin at the plasma membrane, by accelerating both its endocytosis and exocytosis.

5.3.4. The regulation of nephrin at the plasma membrane by PACSIN2 is independent of PACSIN2 phosphorylation status at S313

PKCα regulates nephrin endocytosis and PACSIN2 phosphorylation at S313 (89, 316). As we found that PACSIN2 regulates nephrin endocytosis, we hypothesized that this regulation may be dependent on the status of PACSIN2 phosphorylation at S313. To test this hypothesis, we overexpressed S313 phosphomimetic mutants of PACSIN2 and measured the presence of nephrin at the plasma membrane by surface labelling of nephrin using On-Cell Western. PACSIN2-S313E and PACSIN2-S313A mimic the phosphorylated and dephosphorylated forms of PACSIN2, respectively. Surprisingly, however, overexpression of the mutants did not alter the presence of nephrin at the plasma membrane when compared to overexpression of wildtype PACSIN2 (not shown).

5.3.5. Palmitic acid, but not high glucose, regulates the association of PACSIN2 and nephrin

To test which circulating factor elevated in the serum of individuals with diabetes could modulate the trafficking of nephrin in a PACSIN2 dependent manner, we treated SNPs with high glucose, palmitic acid conjugated to BSA or a combination of both and analysed the association of PACSIN2 and nephrin by PLA. Notably, high glucose alone did not increase the association of PACSIN2 and nephrin, but palmitic acid or palmitic acid together with high glucose increased the association by 27% and 32%, respectively (study II, figure 7). Importantly, the change in their association was not due to an increase in either PACSIN2 or nephrin expression levels (study II, supplemental figure 5). These results indicate that circulating FFAs rather than glucose appear to regulate the interaction of PACSIN2 and nephrin.

5.3.6. Rabenosyn-5 is in complex with PACSIN2 and upregulated in the glomeruli of obese ZDF rats

To define which step of nephrin trafficking is regulated by PACSIN2, we immunoprecipitated PACSIN2 from wildtype mouse podocytes and searched for its novel interaction partners by mass spectrometry. Amongst the proteins identified, rabenosyn-5 raised our interest because of its role in endosomal recycling (390). We repeated the immunoprecipitation and used Western blotting to confirm the presence of rabenosyn-5 in the precipitate obtained with PACSIN2 IgGs from lysates of both

SNPs and isolated human glomeruli. We also performed reciprocal immunoprecipitation and detected PACSIN2 in the precipitates obtained with rabenosyn-5 IgGs in lysates of SNPs and isolated human glomeruli (study II, figure 5a, b). We measured the level of expression of rabenosyn-5 in obese and lean ZDF rats and interestingly, we found an increase of its expression in isolated glomeruli from obese rats at both 8 and 34 weeks (study II, figure 5c–e). The increase of rabenosyn-5 in the glomeruli appeared to be due to an increase in both podocytes and other cells types, as shown by double immunofluorescence of rabenosyn-5 and nephrin as a marker of podocytes (study II, figure 5f). These results indicate that PACSIN2 and rabenosyn-5 are upregulated in the glomeruli of ZDF rats and may function together in the intracellular trafficking of nephrin in podocytes.

5.3.7. Rabenosyn-5 overexpression increases the interaction of PACSIN2 and nephrin

To investigate the possible role of the PACSIN2-rabenosyn-5 complex in the trafficking of nephrin, we started by analysing whether the three proteins are expressed in the same cellular compartments. First, we overexpressed PACSIN2-eGFP and found it to colocalise occasionally with both rabenosyn-5 and nephrin in vesicles throughout podocytes, concentrating in the perinuclear area and close to the plasma membrane (study II, figure 6a). Interestingly, rabenosyn-5-eGFP overexpression led to a more abundant colocalisation of the three molecules and triggered an accumulation of both PACSIN2 and nephrin in enlarged vesicles in the juxtanuclear area (study II, figure 6b). Indeed, PLA revealed that overexpression of rabenosyn-5-eGFP increased the association of PACSIN2 with nephrin by 90% (study II, figure 6c, d). The change in the interaction of nephrin and PACSIN2 was not due to changes in their expression levels (study II, supplemental figure 4). Therefore, our results indicate that rabenosyn-5 regulates the association of PACSIN2 and nephrin, and may affect the localisation of PACSIN2 and nephrin in podocytes.

Collectively, we show that PACSIN2, elevated in the glomeruli of ZDF rats, might be responsible for the aberrant localisation of nephrin. This effect apparently involves rabenosyn-5 and could be triggered by the elevated FFAs in the sera of obese ZDF rats.

5.3.8. PACSIN2 alters the actin cytoskeleton organisation in cultured podocytes when dephosphorylated

Finally, we hypothesized that the overexpression of PACSIN2 or its aberrant phosphorylation in DKD could participate in the loss of podocyte function by regulating the actin cytoskeleton. To test this hypothesis, we overexpressed PACSIN2 and its phosphomimetic mutants in proliferating human podocytes and analysed the effects on actin cytoskeleton by immunofluorescence and high-content analysis. The overexpression efficiency of the myc-PACSIN2 constructs is shown in study III, figure 5a. For the high-content analysis, podocytes were fixed and stained with CellMask Blue, Hoechst and tubulin for segmentation purposes, with phalloidin to stain F-actin, and with an IgG targeting myc to select the cells that were positive for PACSIN2 or its mutants. The cells were divided into three groups based on myc staining: no PACSIN2

overexpression, weak overexpression and high overexpression. In terms of the actin cytoskeleton, the cells were divided in “normal” looking cells, having a normal morphology with organised and clear actin stress fibres or “altered” if the actin was disorganised or the overall cell morphology was affected. A third class was used to exclude rounded or dividing cells. Examples of cells classified as “normal” or “altered” are shown in study III, figure 5b. Interestingly, we found that overexpression of PACSIN2-wt lowered the ratio of “normal” to “altered” looking cells, suggesting that PACSIN2-wt overexpression alters the morphology of podocytes and the organisation of their actin cytoskeleton (study III, figure 5c). Compared to the overexpression of PACSIN2-wt, overexpressing the phosphomimicking mutant PACSIN2-S313E showed a limited effect on the proportion of normal looking cells (study III, figure 5c). In contrast, overexpression of the non-phosphorylatable mutant PACSIN2-S313A had a similar effect as PACSIN2-wt and reduced the proportion of cells with disorganised actin cytoskeleton as compared to PACSIN2-S313E (study III, figure 5c). Figure 5d shows a representative heatmap from one replicate of the figure 5C. The heatmap indicates fold changes in morphology-, intensity-, and texture-based features between cells overexpressing myc-PACSIN2-wt/S313E/S313A that were used to separate “normal” and “altered” looking cells (figure 5d). In summary, we found that elevation of PACSIN2 expression alters the actin cytoskeleton organisation in podocytes, and that overexpression of the phosphomimicking mutant PACSIN2-S313E partially prevents it.

5.4. Opposing roles of septin 7 and NMIIA at the docking site of GSVs – study IV

Our laboratory has previously reported that septin 7 interacts with nephrin, hinders GSV trafficking and lowers glucose uptake in podocytes (244). In this part of my thesis project, we aimed at refining the molecular events occurring at the GSV docking and fusion sites at the plasma membrane in the presence or absence of insulin, focusing on molecules interacting with septin 7.

5.4.1. NMIIA is a novel interaction partner of septin 7 in podocytes

Using the same strategy as for PACSIN2, we immunoprecipitated septin 7 from human podocyte lysates and analysed its interaction partners by mass spectrometry. Interestingly, nonmuscle myosin heavy chain (NMHC) II A was detected in the precipitate (study IV, figure 1a). Two NMHCs, two essential light chains and two regulatory light chains (RLCs) form the hexameric nonmuscle myosin complex (391). Importantly, NMIIA was found to regulate the docking and fusion of GSVs in adipocytes (392-394), indicating that it plays a role in the insulin response in another insulin-sensitive cell type. Co-immunoprecipitations followed by Western blotting confirmed that NMHC-IIA and septin 7 are present in the same complexes in cultured human and mouse podocytes. The presence of the complex *in vivo* was confirmed by

immunoprecipitation using isolated rat glomeruli (study IV, figure 1b). In addition, immunohistochemistry with IgGs targeting septin 7 and NMHC-IIA revealed that both proteins are expressed in podocytes in human kidneys (study IV, figure 1c,d). These results suggest that septin 7 and NMIIA function together in podocytes.

5.4.2. NMIIA is present and active in the SNAP23 complex

We have previously shown that septin 7 and nephrin interact in podocytes with the v-SNARE VAMP2 (244). Another study showed that nephrin is required for the full response of podocytes to insulin in glucose uptake (243).

Using SNPs, we examined the interactions of NMIIA and septin 7 with SNAP23 and nephrin. SNAP23 is the t-SNARE responsible for the tethering and docking of GSVs at the plasma membrane (108). Interestingly, after immunoprecipitating nephrin from SNPs, we detected septin 7, SNAP23 and NMHC-IIA in the precipitate by Western blotting (study IV, figure 1j). In line with this, immunoelectron microscopy of rat kidneys revealed that septin 7 is expressed in the foot processes of podocytes, where nephrin is located *in vivo* (study IV, figure 1e,f). Furthermore, immunoprecipitation of SNAP23 followed by Western blotting for septin 7 and myosin complex proteins confirmed the presence of septin 7 and NMIIA, both its heavy (NMHC-IIA) and activated regulatory chain RLC (phosphorylated regulatory light chain, pp-RLC), in complex with SNAP23 in podocytes (study IV, figure 1k). These interactions were confirmed using PLA (study IV, figure 1l–o). Altogether, the interaction studies indicate that septin 7 and NMIIA are in complex with SNAP23, a t-SNARE protein, and nephrin, shown to aid the fusion of GSVs with the plasma membrane (243).

5.4.3. NMIIA knockdown prevents the insulin-stimulated glucose uptake in podocytes

To test whether NMIIA plays a role in glucose entry into podocytes, we knocked NMHC-IIA down using siRNAs and measured the glucose uptake in basal, starved and insulin-stimulated conditions. In basal and starved conditions, the loss of NMHC-IIA did not change the glucose uptake (study IV, figure 2c, d). However, NMHC-IIA knockdown prevented the increase of glucose uptake upon insulin stimulation. Surprisingly, it even reduced the uptake compared to the nonstimulated NMHC-IIA knockdown cells (study IV, figure 2d). This indicates that functional NMIIA complex is required for a normal glucose uptake after insulin stimulation in podocytes.

5.4.4. NMIIA knockdown prevents the interaction of VAMP2 with SNAP23 upon insulin stimulation

As we found that both septin 7 and NMIIA are in complex with the t-SNARE SNAP23, we aimed to dissect the relationship between septin 7 and NMIIA, and their role on the fusion of GSVs. The interaction of the v-SNARE VAMP2 with the t-SNARE SNAP23 is a prerequisite for the entry of glucose into the cells (110). Thus, we measured the interaction of VAMP2 with SNAP23 in starved or insulin-stimulated podocytes, in the presence or absence of NMHC-IIA. In control cells, insulin stimulation successfully

increased the association of VAMP2 with SNAP23, as shown by co-immunoprecipitation and PLA. However, the increase was prevented by knocking down NMHC-IIA (study IV, figure 3). This indicates that NMHC-IIA knockdown prevents the association of VAMP2 with SNAP23, therefore preventing the docking and fusion of GSVs with the plasma membrane after insulin stimulation.

5.4.5. Insulin stimulation lowers the presence of septin 7 and increases the activity of NMIIA in the SNAP23 complex

Next, we investigated the effect of insulin on the presence of septin 7 and NMIIA at the SNAP23-containing docking sites. Co-immunoprecipitations and PLA revealed that insulin reduces the interaction of SNAP23 with septin 7 (study IV, figure 4a–d, g, h, k). On the other hand, insulin did not change the amount of NMIIA present at the docking site as shown by the absence of change in the association of NMHC-IIA and SNAP23 after insulin stimulation (study IV, figure 4c, e). Strikingly, however, the activity of the NMIIA complex, shown by the phosphorylation of the RLC, was increased at the docking site after insulin stimulation, as shown by co-immunoprecipitation and PLA of pp-RLC and SNAP23 (study IV, figure 4c, f, i, j, l). Taken together, these results indicate that upon insulin stimulation, septin 7 is removed from and NMIIA is activated in the SNAP23-containing docking site.

5.4.6. The expression of septin 7 regulates the activity of NMIIA in complex with SNAP23

Next, we aimed to test whether septin 7 knockdown could mimic insulin stimulation at the docking site, as insulin decreases the association of septin 7 with SNAP23. Surprisingly, septin 7 knockdown decreased the presence of the NMIIA complex at the docking site, as shown by the decrease of NMHC-IIA in complex with SNAP23 (study IV, figure 5a, b, d, e, h). However, similar to insulin stimulation, septin 7 knockdown increased the activity of the NMIIA complex present at the docking site, as revealed by the interaction of SNAP23 with pp-RLC (study IV, figure 5a, c, f, g, i). We confirmed that the effect of septin 7 knockdown was specific for the docking site as the expression of the NMIIA complex or its activity at the level of whole podocytes was not changed (study IV, figure 5j–m). Opposingly, overexpression of septin 7 increased the presence of NMIIA in complex with SNAP23 but reduced its activity, as shown by co-immunoprecipitations and PLA (study IV, figure 6). Altogether, the results suggest that removal of septin 7 may be necessary to increase the activity of NMIIA in complex with SNAP23 upon insulin stimulation.

5.4.7. The activity of NMIIA in the SNAP23 complex is increased in diabetic conditions

Next, we tested whether the interplay of septin 7 and NMIIA at the docking site in response to insulin, identified in the *in vitro* experiments, may occur *in vivo* in podocytes in DKD. No difference in the septin 7 expression level was found in the glomeruli of obese Zucker rats compared to glomeruli isolated from the lean ones.

However, Western blotting revealed a decrease of NMHC-IIA expression but an increase in the phosphorylation of the RLC in the glomeruli isolated from obese Zucker rats compared to their wildtype littermate controls (study IV, figure 7).

We then treated differentiated human podocytes for 48 h with sera obtained from male individuals with T1D having normal albumin excretion rate or macroalbuminuria. The treatment did not change the expression level of septin 7 (study IV, figure a, c). However, it did reduce the amount of septin 7 present at the GSV docking sites as shown by PLA between septin 7 and SNAP23 (study IV, figure e–g). Notably, the treatment did not change the expression of NMHC-IIA, but pp-RLC was increased in podocytes treated with sera from individuals with T1D having macroalbuminuria (study IV, figure a, b, d).

Taken together, the results of study IV suggest that in podocytes, removal of septin 7 is necessary for the activation of NMIIA at the docking site of GSVs upon insulin stimulation. Concomitantly, this also allows the interaction of VAMP2 with SNAP23 and nephrin, leading to the increase in glucose uptake (study IV, figure 9).

6. Discussion

6.1. The renal function of the E1-DN mice compared to other rodent models of diabetic kidney disease (study I)

E1-DN mice are hyperglycaemic from the age of two weeks due to the impaired growth of the beta-cell mass and reduced insulin levels (369). In terms of kidney function, homozygous E1-DN mice presented a wide range of features characteristic of DKD, such as increased urine volume and significant albumin excretion. Interestingly, the AER correlated with the hyperglycaemia. Also, albuminuric E1-DN mice developed significant glomerular alterations that are rarely found in rodent models. In particular, the thickening of the GBM is an early feature of human DKD and is found in E1-DN mice with severe albuminuria. Functionally and structurally, the renal defects observed in E1-DN mice are comparable to the other mouse models having defects in the production of insulin. OVE26 mice overexpress calmodulin in the pancreatic beta-cells and develop diabetes, high albuminuria and glomerulosclerosis (288). Akita mice also harbour defects in the insulin production due to a mutation in the insulin 2 gene (277). In the C57BL/6 background, in which they were originally generated, Akita mice develop only moderate DKD phenotype. The phenotype became similar to OVE26 and E1-DN mice when Akita mice were backcrossed to the FVB background, shared by these two other models, highlighting the variability of the renal phenotype in various genetic backgrounds in mice (395).

Induction of diabetes with STZ is a widely used experimental model in both mice and rats, and results in a significant renal phenotype. However, it cannot be excluded that STZ has intrinsic nephrotoxic properties. Thus, an advantage of the transgenic mouse models is the absence of possible nephrotoxic effects of the drug. Db/db and ob/ob mice are also commonly used models for diabetes and its complications. Conceptually, db/db and ob/ob mice are quite different from the E1-DN mice. E1-DN mice are insulin deficient whereas db/db and ob/ob mice become rather insulin resistant (260), suggesting that the models may be useful for studying different aspects of diabetes and DKD. Moreover, even though db/db mice are hyperglycaemic, they develop a rather mild albuminuria and limited glomerular defects (269). Also, the level of albuminuria varies depending on the background and also the colony from which the mice originate, possibly due to genetic drifting (260).

In terms of podocyte function, E1-DN mice showed clear signs of injury. We observed that the homozygous group has a higher rate of glomerular apoptosis than the control group, the lost cells apparently being podocytes. The homozygous mice also had decreased expression of nephrin, although we cannot conclude whether the reduction of nephrin expression is directly due to the hyperglycaemia or rather a consequence of the increased apoptosis of podocytes observed in the homozygous mice. Nevertheless, the fact that other proteins decisive for podocytes function, such as podocin and CD2AP, did not change, support the specificity of the loss of nephrin. Previous studies have also shown that podocin and CD2AP do not change in DKD (233, 396), supporting

our finding. Interestingly, similar loss of nephrin expression is observed in STZ-induced diabetes and also in renal biopsies of individuals with DKD (231, 233). In line with the potential role of nephrin as a driving factor towards the development of DKD, other studies have revealed mislocalisation of nephrin instead of its downregulation (study II, (234)). Therefore, defects in nephrin expression or localisation may be a specific change occurring in DKD.

The main limitation for the experimental use of the E1-DN mice is the variability of the phenotype amongst the homozygous mice. Some individuals develop severe hyperglycaemia, albuminuria and glomerulosclerosis, whereas the level of hyperglycaemia and albuminuria in some homozygous mice remains the same as in the wildtype mice. This suggest that breeding of a large number of mice and selecting the E1-DN mice with albuminuria may be necessary to study the appearance of advanced features of DKD that we characterised in this study. In addition to this specific weakness of the model, E1-DN mice present the same limitations as the other rodent models for studying metabolic human diseases. In human, diabetes occurs later in life as compared to the hyperglycaemia observed after two weeks after birth in E1-DN mice. Also, the kidney defects in human appear after years of diabetes. The short lifespan of rodents is a limiting factor when studying diseases that are consequences of lifelong metabolic changes in human. Thus, the glomerular changes in the rodent models may not develop to the advanced stage observed in humans, especially since rodents are considered more resistant to renal injury.

Nevertheless, even though the E1-DN mice have their own specific limitations and also the limitations shared with other rodent models of DKD, the homozygous E1-DN mice develop albuminuria and show some advanced glomerular changes that are observed in the human disease. This suggests that the model can be useful for identifying factors regulating the progression to overt DKD or for testing drugs aiming to prevent the progression of DKD towards its advanced stages.

6.2. PACSIN2 and its phosphorylation at S313 in diabetes and diabetic kidney disease (studies II and III)

My thesis project identifies PACSIN2 as a novel protein upregulated in podocytes in DKD. Specifically, we found that the expression of PACSIN2 is increased in the glomeruli of ZDF rats, a model of advanced DKD with severe albuminuria and decreased eGFR (study II and III). On the other hand, no change in its expression was observed in the glomeruli of individuals with diabetes but no deterioration of the renal function (studies III). In developing kidneys, PACSIN2 is strongly expressed in proximal and distal tubules, but only sparsely in the glomerular tuft (333). In adult mice, the expression in the tubules diminishes whereas in the glomeruli it increases, although it still remains at a low level. In line with our results, PACSIN2 expression in the tubular compartment was increased upon ischemia-reperfusion injury (333), suggesting that PACSIN2 is upregulated upon injury, both in tubules and in glomeruli.

We also evaluated whether PACSIN2 phosphorylation is changed in diabetes and DKD. Again, we found that PACSIN2 phosphorylation at S313 is not changed in the glomeruli isolated from people with diabetes having no signs of DKD (study III). However, PACSIN2 phosphorylation at S313 is increased in the glomeruli isolated from obese ZDF rats, as the increase in phosphorylation remains significant when normalising to the elevated total PACSIN2 (study III). Interestingly, culturing podocytes with sera from people with T2D and microalbuminuria increases the phosphorylation of PACSIN2, when compared to podocytes cultured with sera from people with T2D with normal albumin excretion rate. In HeLa cells, PACSIN2 phosphorylation at S313 is mediated by PKCα (316). Importantly, classical PKCs are known enhancers of the progression of diabetic complications, and their systemic activation in diabetes has been reported (196, 197). Thus, the increase in PACSIN2 phosphorylation at S313 is in line with the literature that reports that PKCα is upregulated in the glomeruli of diabetic and albuminuric rats ((397), study III). The elevation of PACSIN2 phosphorylation at S313 in the glomeruli of obese ZDF rats, but not diabetes alone, is also in line with the finding that PKCα participates in the loss of glomerular permselectivity in DKD (398).

The general dogma states that PKC activation is mainly driven by hyperglycaemia (196, 399, 400), and several studies using podocytes in culture report that increased glucose concentration enhances PKC activity (85, 86, 89, 386, 401). We did not examine directly the activation of PKC, but to our surprise, culturing podocytes in medium containing high glucose does not induce the phosphorylation of PACSIN2 at S313 (study III). This raises the possibility that the phosphorylation is triggered by another circulating factor altered in the sera of ZDF rats and individuals with T2D and microalbuminuria. Palmitate is the most abundant FFA in the circulation and is increased in diabetes (388, 389). Interestingly, we found that treating podocytes with palmitate induces a PKC- and time-dependent phosphorylation of PACSIN2 at S313 (study III). These results are supported by previous literature showing that in addition to high glucose, palmitate can induce the activation of PKCs in vascular cells and podocytes (402, 403). Based on genetic and pharmacological studies, inhibition of PKCs has been proposed as a therapeutic approach to treat DKD. Indeed, in STZ-treated mice, PKCα is involved in the loss of glomerular permselectivity by contributing to the loss of nephrin expression (233, 398, 404), and PKCβ participates in the progression of fibrosis in the glomeruli (404-406).

The mechanisms by which the FFAs mediate their deleterious effects in podocytes remain unclear. In culture, palmitate treatment induces insulin resistance in podocytes, supporting its potential role in the progression of DKD (407). Podocytes express FFA receptors and the receptors mediate FFA-induced micropinocytosis (408). In turn, palmitate treatment triggers endoplasmic reticulum stress, mitochondrial superoxide generation and increases autophagic flux (403, 409, 410). Studies in other cell types confirm the activation of these cellular mechanisms and suggest that small G-proteins and PKC are involved (411-414). *In vivo*, high-fat diet induced elevation of serum FFAs increases the susceptibility to glomerular injury (408). Also, the correlation of the GFR with the expression of genes involved in the lipid metabolism suggests an association of the progression of DKD with a change in

the lipid profile in humans (415). In other systems, palmitate induces insulin resistance in hypothalamic cells and impairs insulin secretion in β-cells (411, 413).

This thesis work reveals PACSIN2 as a protein whose phosphorylation is increased in the glomeruli in DKD, likely due to the elevated activity of PKCα (study III). This raises the possibility that PACSIN2 could mediate some of the pathogenic effects of PKCα activation in the glomeruli in DKD.

6.3. Role of PACSIN2 in the trafficking of nephrin (study II)

PACSIN2 is involved in the trafficking of receptors, such as EGF-R, mannose-6-phosphate receptor and transferrin receptor, and transmembrane proteins, such as VE-cadherin (292, 294, 320, 331, 345). In study II, we found that PACSIN2 regulates the expression of nephrin at the plasma membrane of podocytes, as overexpression of PACSIN2 decreases the presence of nephrin at the cell surface but increases its turnover. We observed that PACSIN2 and nephrin colocalise in various compartments of the cell, including the plasma membrane, endosomes and the perinuclear area (study II).

Previous studies show that nephrin is internalised by various endocytic pathways, including CDE and CIE (84-86, 89). In line with this, our live-cell TIRF microscopy experiment indicates that nephrin enters the cells in various submembrane regions, some of which are large and stable PACSIN2-eGFP-positive structures, some are smaller and more dynamic domains positive for PACSIN2-eGFP, and some are devoid of PACSIN2 (study II). The rational for the choice between the different pathways of nephrin internalisation may be physiological or pathological adaptation to the changing glomerular environment (84). In particular, CDE was proposed as a constitutive and rapid pathway, whereas raft-mediated CIE a slower pathway activated by stimuli that trigger the phosphorylation of nephrin (84). The raft-mediated pathway was suggested to be activated in conditions requiring internalisation of a large quantity of nephrin molecules, such as during development or upon injury (84). Therefore, PACSIN2 could participate in the internalisation of nephrin *via* various pathways, as PACSIN2 is known to regulate different endocytic pathways (292, 301, 321). This is supported by the various patterns of nephrin internalisation, with or without PACSIN2, observed by the live-cell TIRF imaging, as well as by the partial colocalisation of both PACSIN2 or nephrin with clathrin or caveolin-1 (study II, study II supplemental data). However, our experiments did not reveal whether PACSIN2 inhibits or enhances the endocytosis of nephrin through either CDE or CIE, or whether PACSIN2 targets nephrin towards a specific pathway.

PKCα, known to phosphorylate PACSIN2 at S313 (316), also increases nephrin endocytosis in hyperglycaemic conditions (89). For this, it phosphorylates human nephrin at T1120/1125, leading to the interaction of nephrin with β-arrestin2 (86). Thus, we tested whether PACSIN2 phosphorylation at S313 participates in the regulation of the internalisation of nephrin. Surprisingly, however, we could not detect

any differences in the expression level of nephrin at the surface of podocytes after overexpression of PACSIN2-S313E/A mutants, as compared to overexpression of PACSIN2-wildtype (data not shown).

In an attempt to further define the mechanisms whereby PACSIN2 regulates the intracellular trafficking of nephrin, we immunoprecipitated PACSIN2 and performed a mass spectrometry analysis of the isolated complexes. We identified rabenosyn-5 as a novel interaction partner of PACSIN2 in cultured podocytes, and confirmed their interaction also in human glomeruli isolated from nephrectomy samples (study II). Rabenosyn-5 is a rab5 and rab4 effector involved in endosomal recycling (390). Interestingly, rabenosyn-5 expression is elevated in the glomeruli of obese and albuminuric ZDF rats (study II). Moreover, rabenosyn-5, PACSIN2 and nephrin colocalise *in vitro* and overexpression of rabenosyn-5 increases the interaction of PACSIN2 and nephrin, apparently by trapping them in the peri-nuclear compartment (study II). Previous work established that PACSIN2 interacts with EHD proteins, known regulators of endosomal trafficking that associate with the actin cytoskeleton (324, 325, 416). Together, PACSIN2 and EHD proteins function in the nucleation and scission of tubular recycling endosomes from rabenosyn-5 positive early/sorting endosomes (319, 325). In line with this, we found that PACSIN2 decorates the early endosomal compartments where nephrin is located shortly after its internalisation and that a significant amount of internalised nephrin is recycled back to the plasma membrane (study II, study II supplemental data). As opposed to previous literature, which mainly attributes the increased internalisation of nephrin in DKD to hyperglycaemia (85, 86, 89), we found that palmitate, but not high glucose, changes the amount of nephrin in complex with PACSIN2 (study II).

Collectively, we identify PACSIN2 as a novel regulator of nephrin trafficking in podocytes, likely functioning together with EHD proteins and rabenosyn-5 in the endosomal compartment. Mechanistically, the elevated circulating FFAs increase the interaction of PACSIN2 with nephrin, possibly enhancing the pace of intracellular trafficking of nephrin. However, it remains to be established whether PACSIN2 directs nephrin towards a specific endocytic pathway and how the internalisation of nephrin is regulated by PACSIN2 in disease states.

6.4. Role of PACSIN2 in the regulation of the actin cytoskeleton (study III)

Parameters defining the organisation of the actin cytoskeleton and the general cell morphology have previously been used in high-content screening studies aiming to evaluate the toxicity of various small molecules for podocytes (417). The approach has been validated by using puromycin aminonucleoside, known to induce loss of actin stress fibres and death of podocytes (417). Notably, we found that overexpression of PACSIN2 alters the actin cytoskeleton organisation and general morphology of podocytes (study III). This suggests that upregulation of PACSIN2 in DKD may be harmful for podocytes, although the overexpression levels obtained in culture are higher than what was observed in the glomeruli in DKD (study II and III).

Furthermore, high-content screening analysis reveals that the effect is stronger when PACSIN2 is dephosphorylated, as overexpression of PACSIN2-S313E induces a milder effect as compared to overexpression of PACSIN2-wildtype or -S313A (study III).

Originally, PACSIN2 was described to function in the regulation of the actin cytoskeleton and endocytosis (292, 294, 328). In particular, PACSIN2 interacts with N-WASP and overexpression of PACSINs induces filopodia formation in an N-WASP-dependent manner (294, 328). N-WASP is an actin nucleator and its knockout in podocytes in mice results in proteinuria at the age of three weeks (57). Electron microscopy analysis of the glomeruli of the knockout mice reveals accumulation of actin patches and disruption of podocyte foot processes (57). This highlights the importance of a properly regulated actin cytoskeleton in podocytes, and raises the question whether the upregulation of PACSIN2 plays a role in the progression of DKD by affecting the actin organisation, possibly by altering the localisation of N-WASP (328). In addition to N-WASP, knocking out a plethora of actin regulators in podocytes results in albuminuria and FSGS-like phenotype, strengthening the relevance of actin for this cell type. Such proteins include the small GTPases RhoA (44, 45), Rac1 (46, 49) and Cdc42 (46, 47). Specifically, Rac1 knockout in podocytes worsens the hypertension-induced kidney damage but improves resistance of podocytes to protamine sulphate-induced damage (46). In the case of DKD, the literature is conflicting as knockout of Rac1 was reported to either improve or worsen STZ-induced kidney injury (50, 51).

Interestingly, PACSIN2 regulates the activity of Rac1 (305, 329) and this relies on the phosphorylation of PACSIN2 phosphorylation at S399, the site phosphorylated by casein kinase 2 (329). However, little information is available about casein kinases in DKD, in which they have been linked to damage of mesangial cells rather than podocytes (418, 419). Thus, it is not possible to speculate whether this site could participate in the regulation of the actin cytoskeleton in podocytes in DKD.

In my thesis, we establish S313 as an actin-regulating phospho-site for PACSIN2 in podocytes (study III). The phosphorylation of PACSIN2 at S313, apparently carried out by PKCα, is likely more relevant than the phosphorylation at S399 for podocytes, as PKCα has been shown to promote the progression of DKD (233, 398, 404). Indeed, our results suggest that the function of PACSIN2 is disturbed in podocytes, that PACSIN2 is a target of PKCs in podocytes, and that the phosphorylation status of PACSIN2 can alter the actin cytoskeleton organisation. Previously, the kinase activity of PKC was already found to regulate the function of actin regulators in podocytes (420, 421). As opposed to this literature, indicating that PKC mediates the deleterious effect on the actin cytoskeleton (420, 421), our *in vitro* experiment suggests that constitutively phosphorylated PACSIN2 has a milder effect than wildtype or non-phosphorylatable PACSIN2 on the actin cytoskeleton organisation. At this point, it is difficult to estimate the effect of PACSIN2 on the actin organisation of podocytes in DKD *in vivo*, as both its expression and phosphorylation at S313 are increased in the glomeruli in DKD. Therefore, the differences of the models make it difficult to speculate whether the beneficial effect of PACSIN2 phosphorylation at S313 prevails over the deleterious effect of total PACSIN2 increase in podocytes in DKD. More experiments using PACSIN2 knockout and S313 mutant knockin mice are necessary to test the relevance

of PACSIN2 and its phosphorylation in the maintenance of the actin cytoskeleton *in vivo* and in the context of DKD.

6.5. Septin 7 and NMIIA in the trafficking of GLUT4 in diabetic kidney disease (study IV)

In study IV, we refine the mechanisms by which septin 7 exerts its effect on glucose uptake in podocytes, focusing on the GSVs docking and fusion with the plasma membrane. Indeed, podocytes are insulin-sensitive cells and their insulin sensitivity is necessary for kidney function (7, 241). As in other insulin-sensitive tissues, the activation of the insulin signalling pathway in podocytes results in the fusion of GSVs with the plasma membrane (102). This requires an interaction of a v-SNARE with a t-SNARE, and in podocytes, VAMP2 and syntaxin 4 were previously identified as the v- and t-SNAREs involved in GSV trafficking (243, 244). In addition, nephrin is required for the full responsiveness of podocytes to insulin by interacting with the v-SNARE VAMP2 on GSVs (243). Also CD2AP, known to link nephrin to the actin cytoskeleton, regulates the formation of the insulin-sensitive GSV pool (26, 245).

Previously, our laboratory found that septin 7 regulates glucose uptake in both basal and insulin-stimulated conditions, as septin 7 knockdown or depolymerisation of septin filaments lowered the entry of glucose into podocytes and rat fibroblasts stably overexpressing the insulin receptor (244). This effect in podocytes appears to rely on the interaction of septin 7 with VAMP2, nephrin and CD2AP (244). In study IV, we identify NMIIA as a novel interaction partner of septin 7. However, as opposed to septin 7 (244), NMIIA appears to positively regulate glucose uptake in podocytes, suggesting that septin 7 and NMIIA may have opposite functions in podocytes (study IV, (244)). Based on the general literature on NMII, it is possible that NMIIA regulates various steps of GSVs trafficking through regulation of the actin cytoskeleton, such as the approach of GSVs to the plasma membrane, or their tethering or docking and fusion with the plasma membrane (422).

Interestingly, we found that septin 7 and NMIIA interact with SNAP23 (study IV), suggesting that septin 7 and NMIIA regulate the very last steps of the insulin signalling pathway leading to glucose uptake: the docking and fusion of the GSVs with the plasma membrane. This is supported by previous studies showing that NMIIA is activated upon insulin stimulation in adipocytes and is necessary for the final steps of GLUT4 trafficking in muscle cells (423). Supporting a role for SNAP23 as a t-SNARE in podocytes, SNAP23 has been described as a t-SNARE necessary for the glucose uptake in adipocytes and fibroblasts (424-426).

To refine the interplay between septin 7 and NMIIA in the presence or absence of insulin, we used interaction studies such as immunoprecipitation and PLA, the latter of which has recently been confirmed as a method of choice to study the docking and fusion stages of GSV trafficking (427). As expected, insulin increased the interaction of the v-SNARE VAMP2 with the t-SNARE SNAP23, but this effect was prevented by knocking down NMHC, resulting in the absence of insulin-stimulated glucose uptake.

Interestingly, insulin enhanced the activity of NMIIA (study IV), as shown by the phosphorylation of the RLC (428). In line with this, knocking down NMIIA or inhibiting myosin activity in adipocytes prevented the insulin-stimulated glucose uptake (392-394).

We also found that concomitant with the increase in NMIIA activation, insulin stimulation diminishes the amount of septin 7 at the docking site. Similarly, knocking down septin 7 reduces the presence and increases the activity of NMIIA at the GSV docking site (study IV). This raises the question of a possible competition between the function of septin 7 and NMIIA at the docking site. In other cell types, it is known that septins and myosins can either function together or compete, and that they can regulate each other (429, 430). Using purified proteins, it has been shown that septin 9, which is in complex with septin 7 in podocytes (244), inhibits the actin-dependent activity of myosin V (429). Opposingly, in Chinese hamster ovary cells, septin 2 acts as a scaffold for NMII and its kinases, leading to the phosphorylation and activation of its RLC (430).

In podocytes, the mechanism by which septin 7 lowers the activity of NMIIA at the docking site remains to be established. It is possible that septin 7 acts as a scaffold for a phosphatase, which remains to be identified, or simply blocks the access of a myosin kinase to the RLC. The activity of NMIIA is likely necessary for the movement of the GSVs along the actin filaments, but a possible additional effect on the actin cytoskeleton should not be ignored. Indeed, NMIIA has been linked with the reorganisation of F-actin at the cell surface in adipocytes upon insulin stimulation, and actin reorganisation is necessary for the final steps of GLUT4 trafficking from the submembranous space to the plasma membrane prior to the fusion in adipocytes and muscle cells (423, 431). Moreover, knocking down NMHC induced disruption of actin stress fibres in podocytes, supporting the importance of NMIIA for the actin cytoskeleton organisation in this cell type (432). Recently, septins has been proposed to affect the endosomal system dynamics by stabilising the actin network (354). In this model, upon activation of G-coupled receptors, septins would be removed from actin filaments allowing actin remodelling proteins to exert their function necessary for the movement of endosomes (354). It is possible that septins, when at the docking site region, could prevent both myosin activation and actin remodelling by preventing the access of other proteins to their substrates. Taken together, our results indicate that upon insulin stimulation, septin 7 is depleted from the GSV docking site at the plasma membrane, releasing its inhibitory effect on NMIIA, which induces the entry of glucose into the cell.

An aspect that requires further research in the future is the importance of nephrin in this process. Nephrin has been shown to enhance the insulin-stimulated glucose uptake in podocytes, but GLUT4 was found in various plasma membrane domains of podocytes where nephrin is not expressed (241, 243). Therefore, it is likely that nephrin regulates GLUT4 trafficking only in the slit diaphragm region and other proteins are involved in other subdomains of podocytes. Interestingly, we show that both septin 7 and NMIIA interact with nephrin (study IV), but we did not examine whether nephrin was necessary for the effect of NMIIA or septin 7 on glucose transport. Knockdown of septin 7 was able to increase glucose uptake into podocytes without nephrin, indicating

that septin 7 at least has an effect on glucose uptake independently of nephrin (244). However, it remains open whether the effects of septin 7 and NMIIA on GSV docking and fusion is enhanced by the presence of nephrin. Interestingly, the identification of NMIIA as a novel interaction partner of nephrin suggests that defects in nephrin trafficking or expression could play a role in *MYH9*-related disorders, or syndromes involving mutations in the *MYH9* gene, encoding NMHC-IIA. Mutations in *MYH9* can lead to glomerulopathy and proteinuria in human, and podocyte-specific deletion of NMHC-IIA induces albuminuria and glomerulosclerosis in mice (433-435).

In rats with STZ-induced diabetes, the myosin light chain kinase, which phosphorylates the RLC, is increased in the kidneys (436). In line with this, we found that the activity of NMIIA is increased in the glomeruli isolated from obese Zucker rats as compared to the glomeruli isolated from their lean littermates. We also treated podocytes with sera obtained from people with T1D having normal albumin excretion rate or macroalbuminuria. Interestingly, podocytes treated with sera from people with albuminuria have elevated amounts of activated NMIIA and lower level of septin 7 at the docking site of GSVs (study IV). A role for NMIIA in the development of DKD is further supported by the association of polymorphism in *MYH9* gene with DKD in Americans of European origin (437), and the downregulation of *MYH9* expression in the glomeruli of mice and human with DKD (438).

Another point that requires attention in the future is the functional significance of the regulation of glucose uptake by septin 7 and NMIIA in podocytes in DKD. It is clear that functional insulin signalling is required for podocytes (7), but abnormally elevated glucose content in the cytoplasm is generally considered harmful (196). This is supported by a study showing that mice with podocyte-specific deletion of GLUT4 were protected from DKD (439). Also, the expression of septin 7 has been reported to be increased in DKD and to mediate podocyte apoptosis in response to hyperglycaemia (440). Thus, further studies are necessary to define whether changes in the expression level of septin 7 or in the activity of NMIIA, likely leading to changes in glucose uptake into podocytes in DKD, are protective mechanisms or drivers of the progression of the disease.

In summary, we propose a model in which septin 7 inhibits the glucose uptake into podocytes in steady state, possibly by forming a physical barrier or inhibiting actin reorganisation. This prevents the formation of the SNARE complex and subsequent fusion of the GSVs with the plasma membrane. Upon insulin stimulation, septin 7 is released from the complex and this allows the activation of NMIIA. Thereafter, the SNARE complex can assemble and trigger the docking and fusion of the GSVs with the plasma membrane, allowing the entry of glucose into podocytes.

6.6. PACSIN2, septin 7 and NMIIA: intertwined functions in actin cytoskeleton organisation and intracellular trafficking? (studies I–III)

The actin cytoskeleton plays an essential role in several aspects of podocytes physiology (5). As discussed in this thesis, actin, as the main component of the cytoskeleton in

podocytes, is necessary for the maintenance of the highly organised 3D architecture of podocytes. Also, a fully functional intracellular trafficking system, both exocytosis and endocytosis, is crucial for podocytes in order to adapt to the changes in intraglomerular pressure and maintain the composition of the slit diaphragms. In particular, the presence and function of caveolae in podocytes *in vivo* is little explored but may be of critical importance. Caveolae and clusters of caveolae in rosettes are considered as a stock of plasma membrane that can be used by the cell to cope with sheer stress and deformations, and are proposed to be anchored to the actin network (441). Changes in these cellular functions relying on actin may participate in the progression of DKD and actin has therefore been suggested as a target for slowing down the progression of the disease (235).

Interestingly, the functions of PACSIN2, septin 7 and NMIIA are either directly involved in actin reorganisation (PACSIN2) or likely change the local actin dynamics (NMIIA and septin 7). In study III, we show that PACSIN2, as established long ago in other cell types (292, 294), has a direct effect on actin cytoskeleton, and can therefore change the morphology of podocytes. Furthermore, PACSIN2 is a regulator of endocytosis and intracellular trafficking, and in study II, we found that it can regulate the presence at the plasma membrane and the turnover of nephrin, which is possibly the most fundamental component of the slit diaphragm. The role of PACSIN2 in intracellular trafficking is presumably due to both the capacity of PACSIN2 to bend membranes, but also likely to its capacity to reorganise the actin cytoskeleton together with N-WASP, allowing the movement of vesicles. Due to the capacity of PACSIN2 to interact with caveolae and actin, it has been speculated that PACSIN2 is one of the candidate proteins for the anchorage of the caveolae to the actin network (441).

In study IV, we propose that insulin stimulation triggers the activation of NMIIA by depleting septin 7 from the GSV docking site. The release of the inhibition of NMIIA activity, induced by insulin, is also likely to be a prerequisite for the movement of GSVs along the actin filaments during their approach to the plasma membrane. In addition, the literature suggests another effect of NMIIA on local actin reorganisation at exocytosis sites (423, 431, 432), occurring apparently *via* N-WASP (423). The function of septin 7 is also associated with the regulation of N-WASP activity in the context of bacterial internalisation and autophagy (442, 443). Thus, N-WASP appears as a potential central point in the regulation of actin and intracellular trafficking in podocytes, whose function may be regulated by PACSIN2, septin 7 and NMIIA. However, further studies are needed to understand further the relationship between these proteins and N-WASP, and to investigate the potential impact of such interplay for podocyte physiology.

7. Conclusion and future prospects

Pathological changes in podocytes and glomeruli drive the progression towards DKD. In this work, we show that homozygous E1-DN mice with albuminuria present advanced glomerular defects typical of human DKD. We also identified PACSIN2 as a novel regulator of intracellular trafficking and actin cytoskeleton organisation in podocytes, and our work suggests that its function is altered in DKD. Finally, we found that septin 7, together with NMIIA, regulates the final stages of GLUT4 trafficking in podocytes, a mechanism apparently dysregulated in DKD.

Here, we observed that secondary to hyperglycaemia, E1-DN mice develop albuminuria, although inter-individual variation was detected. Nevertheless, individuals with substantial hyperglycaemia developed advanced changes characteristic of DKD, including advanced features that are not often observed in other murine models of diabetes and DKD. However, increasing the consistency of the phenotype of the homozygous mice would be necessary to limit the number of animals used for further studies. It could be achieved by crossing individuals that develop hyperglycaemia and excluding the homozygous mice with normal blood glucose. Another alternative would be to feed the mice high-sugar and/or high-fat diet that could increase the number of mice developing hyperglycaemia and subsequent DKD. This would help to make this model more cost-effective and attractive for future studies.

When this work was started, no information was available about PACSIN2 and the other PACSIN family members in the kidney. However, during my studies, PACSIN2 was suggested to participate in repair processes after ischemia-reperfusion injury in the tubular compartment of the kidney (333). We uncovered that PACSIN2 expression is increased in the glomeruli in DKD, and that this could participate in the altered nephrin localisation that can lead to the loss of glomerular permselectivity (figure 8). Moreover, the phosphorylation of PACSIN2 at S313 was also increased in DKD. Importantly, our results suggest that the phosphorylation status affects the capacity of PACSIN2 to perform its well-established function: regulating the actin cytoskeleton (figure 8). The potential importance of this phosphorylation is further supported by the role of PKCs in the progression of DKD. Thus, PACSIN2 appears as one possible mediator of the effect of PKC inhibition, proposed as a strategy to treat DKD, on the maintenance of nephrin expression and prevention of albuminuria.

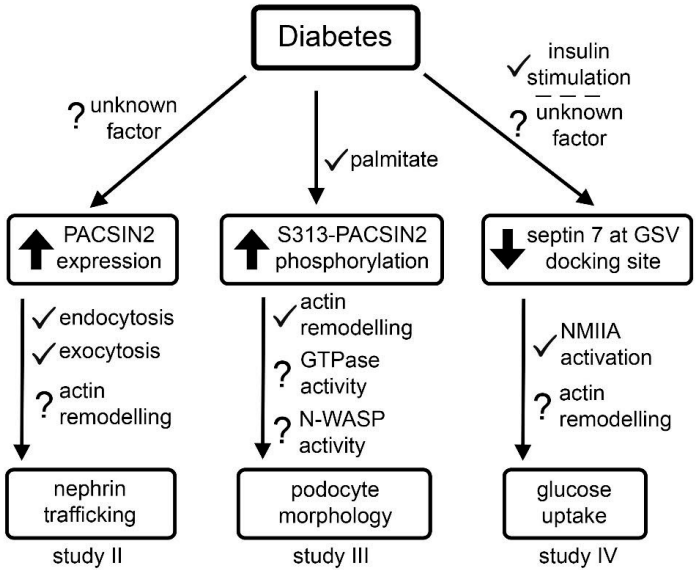

Figure 8: Role of PACSIN2, its phosphorylation at S313 and septin 7 in podocytes under diabetic conditions. "✓" indicates the mechanisms examined in this thesis. "**?**" indicates mechanisms that could be investigated further.

Although we provided novel information on the role of PACSIN2 in podocytes, many questions are without answers. First, PACSIN2 is a cytosolic protein functioning in many organelles *in vitro*, but its intracellular localisation is not yet established in podocytes *in vivo*. Examination of the localisation of PACSIN2 in kidney sections could be carried out using immunoelectron microscopy. Also, we found that palmitate can trigger the phosphorylation of PACSIN2 at S313, but the factor leading to the increased expression of total PACSIN2 in the glomeruli of ZDF rats is still unknown. Moreover, additional work is required to test whether the increase in PACSIN2 and its phosphorylation at S313 accelerate or prevent the progression of the disease. In particular, the use of knockout or knockin mice coupled with induction of diabetes could indicate whether the upregulation of PACSIN2 and its phosphorylation at S313 in DKD are protective mechanisms or drivers of the progression of the disease. These models can also be used to test the implication of PACSIN2 in nephrin trafficking *in vivo*, as novel methods allow the quantification of nephrin at the surface of podocytes *in situ*. Direct analysis of the actin cytoskeleton *in vivo* persists as a challenge, but these models can also be used to measure the expression and activity of actin regulators such as N-WASP and the small GTPases. In culture, overexpression of PACSIN2 and the S313E/A mutants followed by measurements of the activity of these actin regulators will also improve our understanding of the mechanisms by which PACSIN2 regulates the actin cytoskeleton in podocytes.

Our laboratory has previously found that septin 7 regulates glucose uptake in podocytes, but its mechanism of action remained largely unknown. Now, we show that septin 7 is in complex with NMIIA and that these two molecules regulate the docking and fusion of GSVs upon insulin stimulation in podocytes, by affecting the VAMP2-SNAP23 interaction and resulting in glucose uptake (figure 8). Importantly, the activity

of NMIIA was increased in the glomeruli of obese Zucker rats and in podocytes cultured in the presence of sera from individuals with T1D and macroalbuminuria, suggesting that a deregulation of this mechanism occurs in DKD. Further research is necessary to establish to what extent the interplay between septin 7 and NMIIA is relevant *in vivo*. In particular, it will be important to define whether inhibition or activation of NMIIA activity would be a suitable therapeutic strategy. The identification of the factor(s) inducing the depletion of septin 7 from the GSV docking site *in vivo* may also provide new targets for therapeutic interventions. Also, using podocyte-specific knockout of septin 7 in mice challenged with STZ may reveal the importance of septin 7 in podocytes in the context of DKD.

One striking observation when considering our results and the literature about PACSIN2 and septin 7 is the potential overlap of their functions. The two proteins play a role in intracellular trafficking and the regulation of the actin cytoskeleton. Septin 7 acts as a diffusion barrier underneath the plasma membrane, possibly by preventing turnover in the actin cytoskeleton network. On the other hand, PACSIN2 is likely to participate in the reorganisation of the actin cytoskeleton needed for vesicular translocation as it recruits actin cytoskeleton regulators to cellular membranes. Another function that PACSIN2 and septins share but was not examined in this thesis is ciliogenesis. Indeed, both PACSIN2 and septins are present in cilia in cultured kidney tubular cells, and their knockdown alters the length of cilia (333, 356, 444). Therefore, further studies may provide evidence that the functions of PACSIN2 and septins are intertwined in various cellular processes.

Overall, this thesis presents central information on proteins that regulate the actin cytoskeleton organisation and vesicular trafficking in podocytes. The findings help to establish a comprehensive image of the pathological mechanisms underlying DKD. Additional knowledge on the functions of PACSIN2, septin 7 and NMIIA and the mechanisms whereby they regulate actin cytoskeleton and intracellular trafficking in DKD improves our understanding of the disease, as well as of the loss of function of podocytes observed in DKD. Such knowledge is necessary for the identification of novel therapeutic targets and for developing new treatments preventing the progression of the disease.

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